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Structural and Immunological Studies of Pertussis Toxin: Effect of Toxoiding

**A thesis submitted to the Open University for the degree of
Doctor of Philosophy**

by Sarah Fowler BSc. Hons

November 2001

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Dedication

For my Nana

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Abstract

A detoxified form of pertussis toxin (PT) is used as a component of acellular pertussis vaccines, to prevent whooping cough in children. In this study five commercial PT toxoids, and a series of formaldehyde-treated (0 – 1.00 %, w/v) native and non-toxic mutant PT (PT-9K/129G) preparations were analysed, to investigate the effects of detoxification on the structure and immunogenicity of PT.

Native PT and a non-toxic mutant PT, compared using physico-chemical methods and molecular modelling, were found to be structurally comparable. Analysis of the PT toxoids demonstrated the variation in folding, conformation and subunit association induced by the different chemical methods of detoxification. A good murine IgG response to the toxoids was dependent on the maintenance of local structure such as the integrity of the protective S1 epitope, but molecular aggregation did not appear to be detrimental. Sizing analysis of formaldehyde-treated mutant and native PT preparations, illustrated that both mutant and native PT molecules increased in size and heterogeneity, as a function of formaldehyde concentration. Immunoblotting of the formaldehyde-treated preparations confirmed the presence of covalent inter-subunit cross-linking. Immunological analysis of formaldehyde-treated mutant PT preparations demonstrated that a small degree of aggregation may be beneficial for the immune response in mice, but that increasing amino acid modification, cross-linking and aggregation alters the type of immune response and causes a decrease in the neutralising antibodies. Toxicity assays demonstrated that formaldehyde detoxification of PT is due to modification of both the

A- and the B-domain, and is subject to reversion. Evidence for antibody suppression and enhancement by different amounts of residual active PT was also obtained. The observations made here will help us to understand the complex relationships between antigen structure and immune response in vaccines containing PT.

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Publications and communications

Meetings publications

Fowler, S., Bolgiano, B., Byron, O., Xing, D., and Corbel, M. J. (2000) Pertussis toxin: Effects of formaldehyde on structural properties. Proceedings for the 9th European workshop on bacterial protein toxins. *Int.J.Med.Microbiol.* **290**, A63.

Bolgiano, B., Fowler, S., Turner, K., Sesardic, D., Xing, D.K.-L., Crane, D.T., and Corbel, M.J. (2000) Monitoring of protein vaccine components by CD, fluorescence spectroscopy and size exclusion chromatography. *Dev.Biol.* **103**: 51-59.

Poster presentations

Fowler, S., Ravenscroft, N., Bolgiano, B., Crane, D.T., Corbel, M.J., and Xing, D. (January, 1999) Effect of formaldehyde treatment on the structure of genetically detoxified pertussis toxin. Presented at The Society for General Microbiology Meeting, Warwick, UK.

Fowler, S., Bolgiano, B., Byron, O., Xing, D., and Corbel, M.J. (June, 1999) Pertussis toxin: Effects of formaldehyde on structural properties. Presented at the 9th European Workshop on Bacterial Protein Toxins, Ste Maxime, France.

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Pertussis toxin: Characterising a vaccine component using physico-chemical techniques.

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NAD binding: molecular characterisation by modelling and fluorescence. Presented at

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binding on pertussis toxin structure by computer modelling and fluorescence

spectroscopy. Presented at the 10th European Workshop on Bacterial Protein Toxins,

Durbuy, Belgium.

List of Abbreviations

ACT	adenylate cyclase toxin
ADP	adenosine diphosphate
Alum	aluminium hydroxide
AMBER	Assisted Model Building with Energy Refinement, computer programme
ATP	adenosine triphosphate
AUC	analytical ultracentrifugation
BCA	bicinchoninic acid
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CD	circular dichroism
CHO	chinese hamster ovary
CT	cholera toxin
TT	molar circular dichroic absorption
DMSO	dimethylsulphoxide
DNT	dermonecrotic toxin
DT	diphtheria toxin
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
DTP	combined diphtheria tetanus and pertussis
DTT	dithiothrietol
FA	formaldehyde

FAC	fluorescein caproic acid
FCS	foetal calf (or bovine) serum
FHA	filamentous haemagglutinin
Fim2	serotype-2 fimbriae
Fim3	serotype-3 fimbriae
Fmax	wavelength of maximum fluorescence
G_{iα} subunit	inhibitory α-subunit of guanyl nucleotide binding protein
G-protein	guanyl nucleotide binding protein
IC	intracerebral challenge
IFN	interferon
IL	interleukin
IRP	International Reference Preparation
Kd	dissociation constant
T_{ex}	excitation wavelength
T_{em}	emission wavelength
LPC	lysophosphatidylcholine
LT1	heat-labile enterotoxin-1
LT2	heat-labile enterotoxin-2
mAb	monoclonal antibody
MALLS	multi-angle laser light scattering
MHC	major histocompatibility complex
MRC	Medical Research Council
M_{w,app}	apparent weight average molecular weight

NAD	nicotinamide adenine dinucleotide
NATA	n-acetyl tryptophanamide
NMR	nuclear magnetic resonance
PAETA	<i>Pseudomonas aeruginosa</i> exotoxin A
PBS	phosphate buffered saline
PBSG	phosphate buffered saline with gelatin
PDB	protein data bank
PMSF	phenylmethanesulphonyl fluoride
PT	pertussis toxin
PT-9K/129G	genetically detoxified pertussis toxin with the double amino acid substitution: Arg-9 → Lys and Glu-29 → Gly
RCSB	Research Collaboratory for Structural Bioinformatics
SDS-PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
UV	ultra-violet
v/v	volume by volume
VIP-2	vegetative insecticidal protein-2
w/v	weight by volume

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1.1 Overview

Only with the right balance between the immunological efficacy and toxicity of pertussis toxin (PT) will the ultimate whooping cough vaccine be found. PT is an ADP-ribosylating protein toxin secreted by the whooping cough bacteria *Bordetella pertussis*, and is one of the most virulent and immunogenic factors of the bacteria.

Descriptions of the infant disease of whooping cough date back to the 1500s. The causative agent, *B. pertussis*, was first described by Bordet and Gengou in 1906 (Bordet and Gengou, 1906). There are many virulence factors produced by the bacteria but it appears that the major systemic effects of whooping cough are primarily due to PT (Pittman, 1984; Pittman, 1979; Weiss and Hewlett, 1986; Monack *et al.*, 1989). Despite the success of the whole cell vaccine in reducing mortality of the disease, uncertainty about the safety and effectiveness of the whole cell vaccine (Romanus, Jonsell, and Bergquist, 1987; Fine and Clarkson, 1987; Fine and Clarkson, 1982) has urged the development of more defined vaccines composed of protective *B. pertussis* antigens, which could be safer and more reliable for mass vaccination. Some methods of testing the efficacy of these vaccines are in use but reliable laboratory tests of protective efficacy are still being developed.

PT is the primary component of all current acellular pertussis vaccines. High-resolution structures of PT (Stein *et al.*, 1994a; Stein *et al.*, 1994b; Hazes *et al.*, 1996) show that it is of the AB class of protein toxins and the functions of its domains in the host have been described (Locht and Antoine, 1999). Many important biological and immunological properties have been observed (Munoz, 1985), although their complex mechanisms continue to be investigated. Studies on the effects of toxoiding PT by genetic mutation and chemical treatment have assessed modifications to some toxic properties of PT, cell binding activities and a small number of T- and B-cell epitopes (Nencioni *et al.*, 1991; Nencioni *et al.*, 1990; Petre *et al.*, 1996; Pizza *et al.*, 1989; Di Tommaso *et al.*, 1994). There is some physico-chemical evidence to suggest that formaldehyde (FA), one of the chemicals used to toxoid PT, causes an increase in the molecular size of PT (Bolgiano *et al.*, 1999). This thesis presents a detailed structural analysis of PT, and investigates the effects of different toxoiding on its physico-chemical, antigenic and immunological properties, with emphasis on the effects of FA treatment.

1.2 Whooping cough

1.2.1 Clinical characteristics

Whooping cough, also known as pertussis, is caused by *B. pertussis* bacteria, and is an acute infectious respiratory disease primarily affecting young children. *B. pertussis*

colonises the tracheal epithelium (Figure 1.1) and produces the disease effects (Gordon and Hood, 1951). The disease has an incubation period, which lasts between seven and 13 days. The initial catarrhal stage lasts for about two weeks and is characterised by nasal congestion, mild fever and cough (Hodder and Mortimer, Jr., 1992). Damage to the respiratory epithelial cells, including the destruction of cilia, can be seen at this stage. As the second, paroxysmal, stage of the disease ensues, the coughing becomes increasingly forceful and the patient may experience ten to 20 or more paroxysms in 24 hours with up to about 30 coughs per spasm (Gordon and Hood, 1951). In infants older than six months, the characteristic whooping noise occurs as the child inhales through the constricted glottis between coughing spasms, the child has difficulty breathing and apnoea is common. At this stage, bronchopulmonary damage includes ciliary destruction, oedema and emphysema, and the extent of this damage is related to the mortality of the disease. In severe cases the patient may suffer from seizures and may develop encephalopathy caused by anoxia of the brain. The paroxysmal stage lasts between one and four weeks before the effects begin to gradually subside as the convalescent period commences. Although the patient is free of *B. pertussis* after the paroxysmal stage, the convalescent period can last one to six months or more before the effects of the disease cease altogether. Complications of the disease may include pneumonia, due to severe damage to the respiratory epithelium, secondary bacterial infections and acute neurological manifestations such as persistent seizures, hemiplegia, paraplegia, decerebrate rigidity, ataxia, aphasia, blindness and deafness (Gordon and Hood, 1951).

The clinical triad for the definition of a positive diagnosis for whooping cough set by the World Health Organisation is paroxysmal cough for 21 or more days and positive culture

for 2 weeks

within 28 days

1.2.1

An epidemic

of a disease

in the 19th

century

In the present

with epidemics

conducted with

The last time

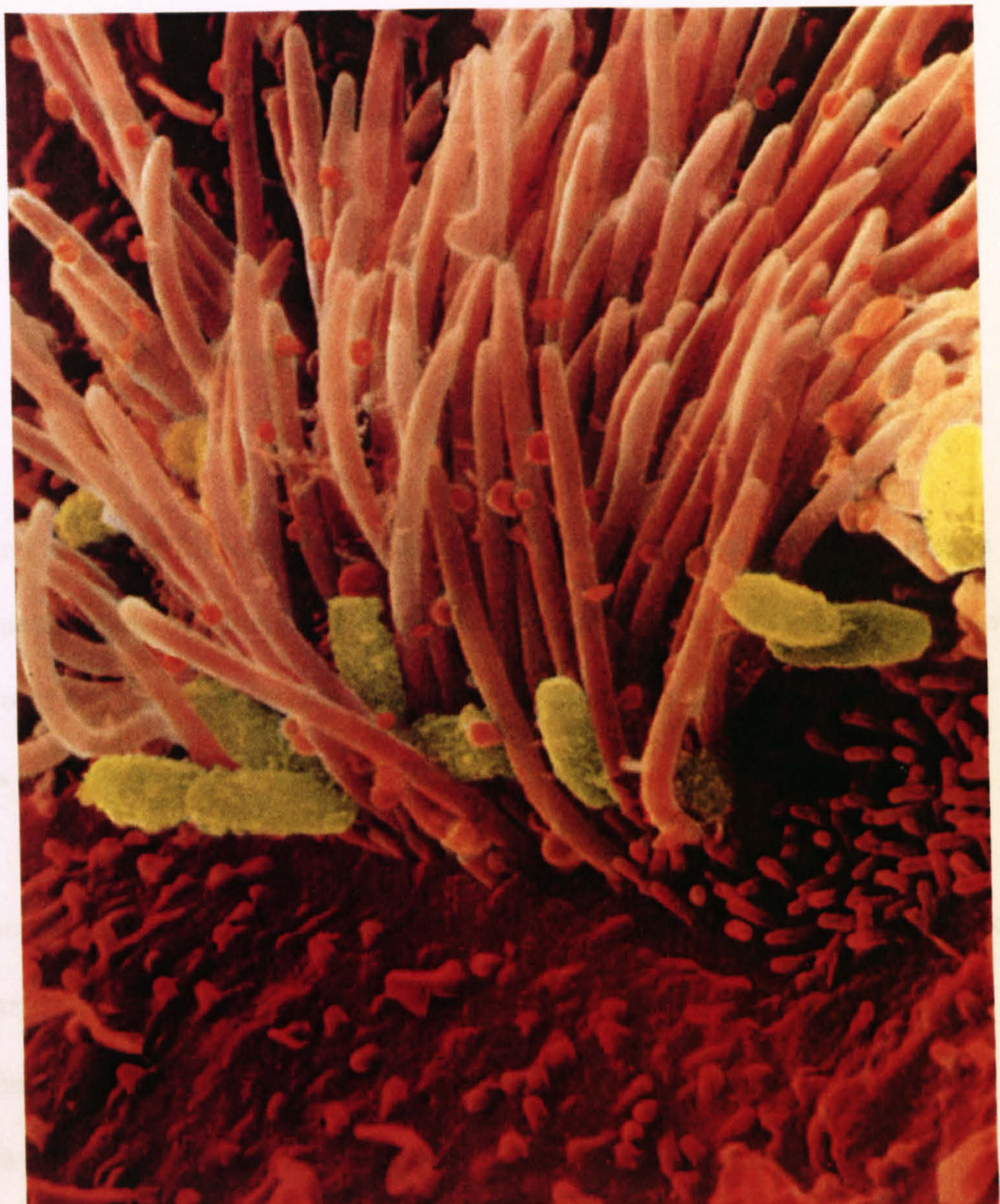


Figure 1.1 *Bordetella pertussis* infection of the tracheal epithelia

Enhanced scanning electron micrograph of live *B. pertussis* cells infecting murine tracheal epithelia. Taken from the NIBSC photographic library.

B. pertussis cells - yellow; cilia - orange; epithelia - red

(Fife and Clarkson, 1982).

In clinical trials the definition of a positive diagnosis for whooping cough set by the World Health Organisation is paroxysmal cough for 21 or more days and positive culture for *B. pertussis*, as well as serologic confirmation, or contact with a culture-proven case within 28 days of the disease (WHO, 1991).

1.2.2 Epidemiology

An epidemic in Paris in 1578, recorded by Guillaume De Baillou, was the first evidence of a disease characteristic of whooping cough (Holmes, 1940). Settlers carried the disease to the New World and there are records of a whooping cough epidemic in Plymouth, Massachusetts in 1649 (Gordon and Hood, 1951).

In the pre-vaccine era, in the USA and in parts of the UK, whooping cough was endemic with epidemics every three to five years. There is evidence that 70 % of children had contracted whooping cough by the age of 17 years (Cherry, Brunell, and Golden, 1988; Fine and Clarkson, 1987; Cherry, 1984). In the USA around 150 cases per 100 000 population were reported per year in the period 1920 – 1945. Since mass vaccination, it has been estimated that this has fallen to about 1 per 100 000 population (Mortimer, Jr. and Jones, 1979; Cherry, 1984). Incidence of whooping cough in the UK is shown in Figure 1.2, and illustrates about a 40-fold decrease in reported whooping cough cases since mass vaccination. However, the epidemic cycle of infection has not changed (Fine and Clarkson, 1982).

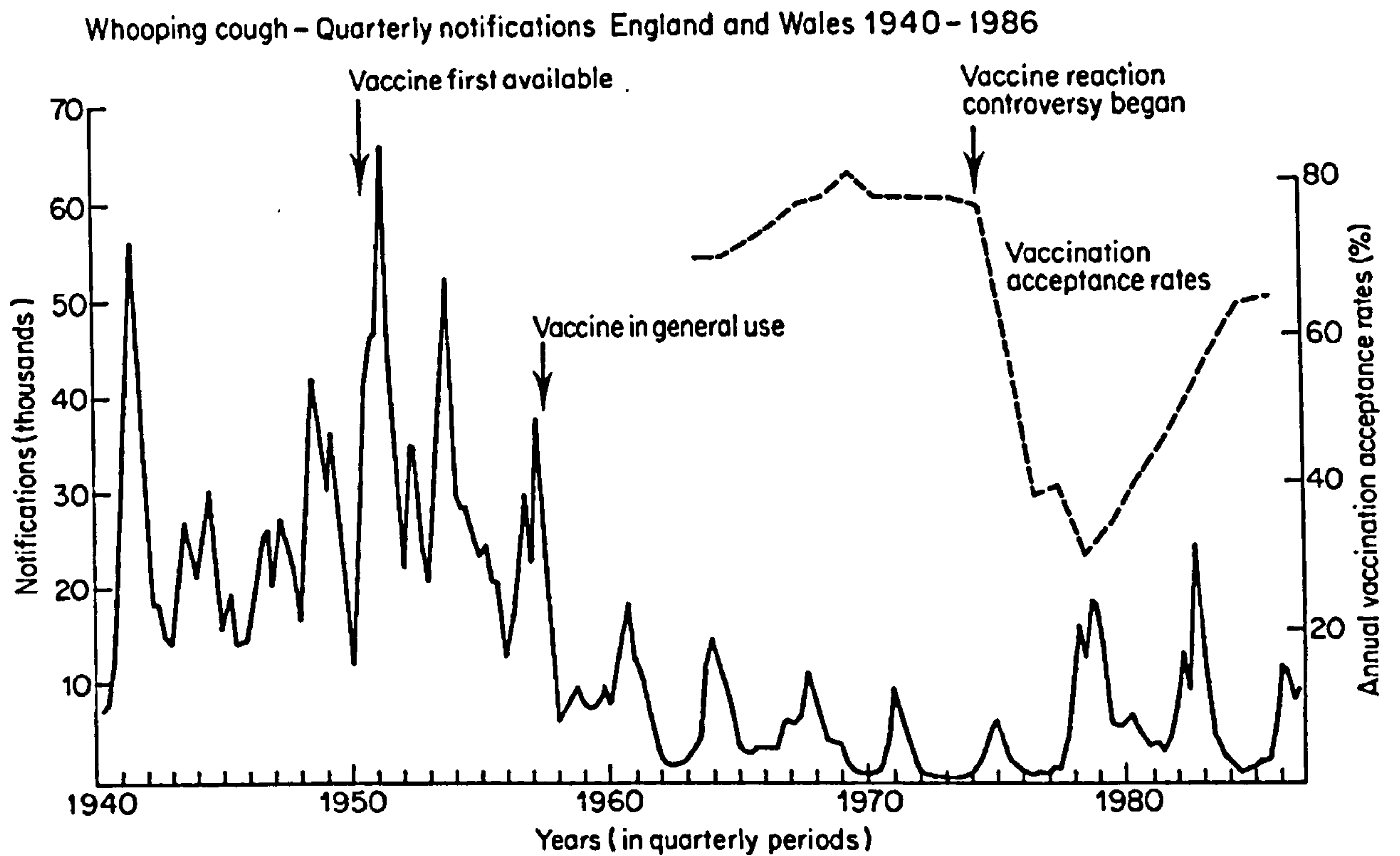


Figure 1.2 Incidence of whooping cough in the UK
 Notifications of whooping cough and annual vaccination rates in England and Wales from 1940- 1986. Taken from Collee and Watt, 1990.

In the USA and in England and Wales the whooping cough epidemic runs in a cycle characteristically peaking every 3 to 4 years (Fine and Clarkson, 1982). Regular cycles of whooping cough infection are determined by the interaction of several factors: the probabilities of infection transmission, rates of recovery from infection and rates of influx of new susceptibles into the population (e.g., by births). An epidemic is initiated when the number of susceptibles reaches a certain threshold level, which can support the increase in disease incidence. Eventually the disease incidence exceeds the rate of influx of new susceptibles and inevitably the numbers of susceptibles decrease until they are below the threshold level. Then the cycle enters an interepidemic phase during which the rate of introduction of susceptibles exceeds the incidence of infection, until the number of susceptibles reach the threshold level once again; and so the cycle continues. Data studied from week 1 of 1950 until week 3 of 1982 showed that the interepidemic periods do not shorten with *B. pertussis* vaccination (Fine and Clarkson, 1982). Therefore, although the incidence of disease is lower due to vaccination, *B. pertussis* is still circulating in the population at the same rate.

1.3 *Bordetella pertussis*

1.3.1 Classification and description

The genus *Bordetella* encompasses four species that cause respiratory tract infections. They all bind to the host's pulmonary ciliated epithelium and produce common pathological effects including, loss of ciliated cells, reduced weight gain of the host, excessive mucus production, a form of cough or sneezing and frequent secondary infection. *B. parapertussis* is responsible for a mild whooping cough-like infection in humans. *B. bronchiseptica* is primarily a pathogen of animals causing kennel cough in dogs and rhinitis in pigs, but is also capable of infecting humans. *B. avium* causes rhinotracheitis of birds and *B. pertussis*, is responsible for whooping cough in humans (Parton, 1989).

B. pertussis is a Gram-negative, non-motile, pleomorphic, coccobacillus (Figure 1.1) measuring 0.2 to 0.8 μm in size. It was first isolated and implicated as the etiological agent of whooping cough in 1906 by Bordet and Gengou (Bordet and Gengou, 1906). *B. pertussis* shares a variety of virulence factors with its related species but is unique in producing PT (Parton, 1989), esteemed the most potent and immunogenic determinant of whooping cough (Weiss *et al.*, 1984; Pittman, 1979; Trollfors *et al.*, 1995).

1.3.2 *B. pertussis* virulence factors and their role in pathogenesis

B. pertussis produces a multitude of virulence factors, ranging from general attachment factors, assisting pulmonary bacterial colonisation, to toxins with highly specific catalytic activity involved in the systemic disease. These virulence factors are co-expressed, and can also be differentially expressed, under the regulation of a virulence locus, which encodes for a regulatory two-component system, Bvg, responsive to environmental stimuli (Kinnear, Marques, and Carbonetti, 2001). The virulence factors of *B. pertussis* are not produced when the bacteria are grown at much under 37 °C, or in certain types of media, and resume their virulent state again under permissive conditions. Phenotypic variation may occur *in vivo* as a method of thriving in the host in an unrecognised carrier-state (Weiss and Hewlett, 1986; Weiss and Falkow, 1984).

The long-accepted mechanism of *B. pertussis* pathogenesis is by means of epithelial attachment, extracellular colonisation and systemic invasion by toxins (Weiss, 1997). An additional mechanism of evading the hosts defences has been discovered in the intracellular growth of *B. pertussis* in macrophages (Ewanowich *et al.*, 1989; Friedman *et al.*, 1992). The major virulence factors of *B. pertussis* are described in the text below and in the accompanying schematic diagram (Figure 1.3).

1.3.2.1 Adhesins

Fimbriae

Fimbriae are proteinaceous filaments that protrude from the surface of bacteria (Figure 1.3). Two types of fimbriae on *B. pertussis*, Fim2 and Fim3, have been described as helical structures (Steven *et al.*, 1986), composed of multiple copies of their homogeneous subunits (~ 20 kDa), distinct for Fim2 and Fim3 (Irons, Ashworth, and Robinson, 1985). The fimbriae are serotype-specific agglutinogens, that is, they have the ability to give rise to specific antibodies capable of agglutinating *B. pertussis* cells (Ashworth, Irons, and Dowsett, 1982; Irons, Ashworth, and Robinson, 1985). They are capable of binding to ciliated cells in the respiratory tract and are believed to play a role in the pathogenesis of whooping cough by aiding *B. pertussis* colonisation (Robinson *et al.*, 1991).

Filamentous haemagglutinin (FHA)

FHA is a cell surface rod-shaped monomeric protein (Figure 1.3) with a molecular weight of 220 kDa and has multiple adhesin properties. It possesses haemagglutinating properties (Sato *et al.*, 1983) and can bind to a variety of cultured cells (Cowell *et al.*, 1986). FHA is involved in colonisation of *B. pertussis* in the respiratory tract by binding to both ciliated epithelial cells (Tuomanen and Weiss, 1985; Tuomanen *et al.*, 1985) and to alveolar macrophages via the integrin CR3 (Relman *et al.*, 1990).

Pertactin

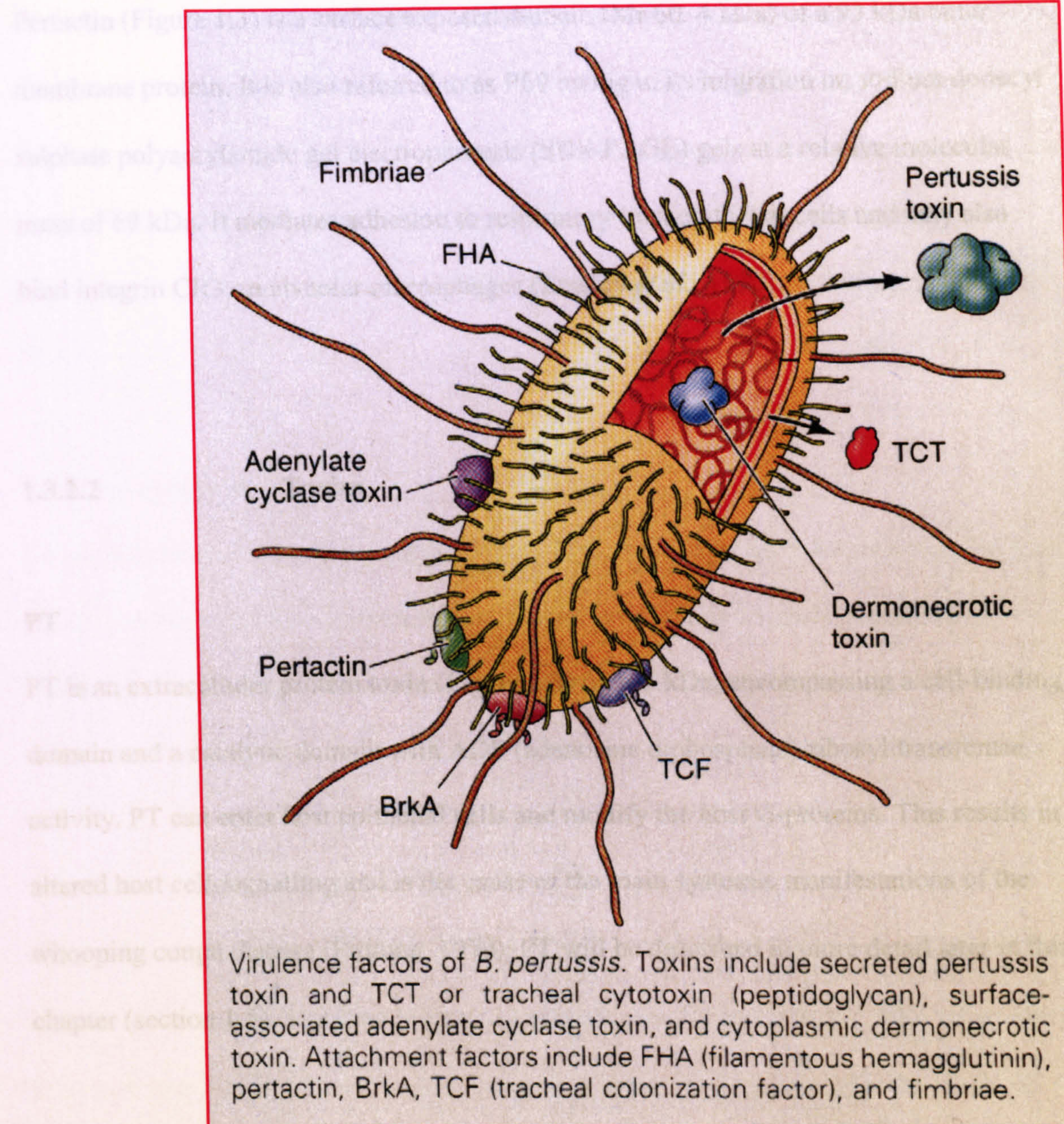


Figure 1.3 Virulence factors of *Bordetella pertussis*

A schematic diagram representing *B. pertussis* and some of its virulence factors. Taken from Weiss, 1997.

Pertactin

Pertactin (Figure 1.3) is a surface exposed domain (Mr 60.4 kDa) of a 93 kDa outer membrane protein. It is also referred to as P69 owing to its migration on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels at a relative molecular mass of 69 kDa. It mediates adhesion to respiratory tract epithelial cells and may also bind integrin CR3 on alveolar macrophages (Emsley *et al.*, 1996).

1.3.2.2 Toxins**PT**

PT is an extracellular protein toxin (Figure 1.3) of 105 kDa, encompassing a cell-binding domain and a catalytic domain with ADP (adenosine diphosphate)-ribosyl transferase activity. PT can enter host epithelial cells and modify the host G-proteins. This results in altered host cell-signalling and is the cause of the main systemic manifestations of the whooping cough disease (Pittman, 1979). PT will be described in more detail later in this chapter (section 1.5).

Adenylate cyclase toxin (ACT)

ACT is an extracytoplasmic protein toxin (Figure 1.3) of 200 kDa which has adenylate cyclase and haemolytic activity (Rogel, Meller, and Hanski, 1991). ACT is required in the initial colonisation of the bacteria (Goodwin and Weiss, 1990). The haemolytic domain allows it to enter many types of eukaryotic cell, perhaps by charge interactions

with lipids (Raptis *et al.*, 1989; Locht and Antoine, 1999). The adenylate cyclase domain is activated by calmodulin and catalyses the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). High levels of cAMP disrupt normal intracellular signalling. ACT can inhibit chemotaxis and superoxide production of human polymorphonuclear leukocytes (Friedman *et al.*, 1987) and induces apoptosis in macrophages, promoting bacterial growth at the site of infection (Khelef and Guiso, 1995).

Tracheal cytotoxin

Tracheal cytotoxin is a small peptide (Figure 1.3), which appears to be secreted, and may be derived from bacterial peptidoglycan. It is not required in the pathogenesis of whooping cough but is specifically toxic to ciliated cells apparently by inhibiting DNA synthesis (Cookson *et al.*, 1989; Cookson, Tyler, and Goldman, 1989; Goldman, 1986).

Dermonecrotic toxin (DNT)

DNT is an intracellular protein toxin (Figure 1.3) of 159 kDa known for producing skin lesions when used to vaccinate laboratory animals (Bordet and Gengou, 1909). It is also known as heat labile toxin since it is completely inactivated by heating to 56 °C for 60 minutes (Livey and Wardlaw, 1984). DNT does not appear to be required for *B. pertussis* virulence in humans although it may be important in the pathogenesis of *B. bronchiseptica* infections in pigs (Roop *et al.*, 1987).

Lipopolysaccharide

Lipopolysaccharide is a stable substance located in the cell envelope of Gram-negative bacteria. Lipopolysaccharide in *B. pertussis* is distinct from that of other Gram negative bacteria in that it is composed of two immunochemically different lipids, lipid A and X, with two different oligosaccharide chains. The lipid X fraction possesses typical endotoxin activities of pyrogenicity and toxicity, while lipid A has potent adjuvant and anti-viral activities (Ayme *et al.*, 1980).

1.4 Pertussis vaccines

1.4.1 *B. pertussis* whole cell vaccine

Soon after the isolation of *B. pertussis* in 1906 attempts were made to produce a vaccine using the attenuated organism (Lapin, 1943). By the 1930's there was evidence from controlled clinical trials that several whole-cell pertussis vaccines could provide statistically significant protection against acquisition and severity of the disease (Kendrick, 1936). The need for an adequate test for vaccine standardisation was met in the 1940's by P. Kendrick *et al.* (Kendrick, Eldering, and Dixon, 1947) who developed the mouse intracerebral (IC) challenge test also known as the Kendrick test. By the 1950s the Medical Research Council (MRC) was able to show that vaccine performance in the

mouse IC challenge test correlated with protection against pertussis in children (Medical Research Council, 1956; Medical Research Council, 1959).

Despite the success in most areas, estimates of whole-cell vaccine efficacy were extremely varied, ranging from 0 to 100 %. This depended on factors such as the study design of efficacy testing but also on real differences in the vaccine preparations (Fine and Clarkson, 1987). For example, studies designed to compare the protective efficacy of different whole cell vaccines illustrated the considerable variation that can arise from vaccines from different manufacturers (Public Heath Laboratory Service Whooping-Cough Committee and Working Party, 1973). As well as differences in methods of attenuation and formulation of the whole cell vaccine, there may also have been differences in the content of virulence factors, which is now known to be affected by the bacterial culture conditions (Lacey, 1960). The whole-cell vaccine was also shown to have a limited duration of protection, exemplified by the common carriage of the mildly symptomatic disease in adults. Due to its disputed efficacy the decision was made to discontinue use of the whole-cell vaccine in Sweden in 1979 (Romanus, Jonsell, and Bergquist, 1987).

The major issue for the whole-cell *B. pertussis* vaccine is that it can have severe side effects, and this has led to waning vaccination in most countries (Golden, 1990; Hodder and Mortimer, Jr., 1992; Miller *et al.*, 1981). There is well established clinical evidence for certain adverse reactions caused by whole-cell pertussis vaccines: local reactions,

such as erythema, tenderness and, less frequently, sterile abscess formation; and systemic reactions including fever, lethargy, anorexia, drowsiness, and prolonged crying (Cherry, 1984; Hewlett and Cherry.J.D., 1997; Cody *et al.*, 1981). All of these responses may not be specific to pertussis vaccine but they occur with more frequency in combined diphtheria, tetanus and pertussis (DTP) vaccine recipients than in recipients of combined diphtheria and tetanus alone (Cody *et al.*, 1981). A significant association was also shown between serious neurological illness and pertussis vaccine, although the cases were few and most children recovered completely (Miller *et al.*, 1981). Moreover some unresolved issues have dealt with the possibility of pertussis being associated on rare occasions with later neurological problems (Miller *et al.*, 1993) and more frequently with acute encephalopathy (Stephenson, 1988).

1.4.2 *B. pertussis* acellular vaccine

The reactogenicity and questionable efficacy of the whole cell vaccine, combined with the realisation that a toxin is involved in the production of whooping cough, (Pittman, 1979), impelled the design of an acellular vaccine (Hewlett and Cherry.J.D., 1997). In the mid 1970s the Hiroko and Yuji Sato first undertook this task in a national effort in the form of the Committee on Improved Pertussis Vaccine controlled by the Ministry of Health and Welfare for Japan (Sato, Kimura, and Fukumi, 1984). They identified two of the most abundant extracellular proteins, now known as FHA and PT, in the growth media of *B. pertussis*. Co-purified vaccines containing these antigens were found to be

protective. In addition, PT and FHA were purified and used in more defined acellular pertussis vaccines (Sato and Sato, 1999; Sato, Kimura, and Fukumi, 1984). PT was treated with FA, to destroy its leukocytosis and histamine sensitising properties, (Sato, Kimura, and Fukumi, 1984). The toxoiding procedure involved FA additions every three days up to a concentration of 0.4 % (w/v) and the mixture incubated at 37 °C for several days. The vaccine was clinically tested and in 1981 it was licensed for use in children of two years old. The endeavours of the committee resulted in the production of acellular pertussis vaccines by six Japanese manufacturers and a triumphant reduction in disease (Sato, 1997), and so a second generation of whooping cough vaccines was born.

Meanwhile, Europe and the United States also started to develop acellular whooping cough vaccines (Rappuoli, 1996). Most manufacturers largely followed the Japanese practice using FA as the detoxifying agent, while some employed other chemicals and included additional *B. pertussis* antigens. There has not been much change in the formulation of acellular vaccines over the years. Today commercial vaccines are minimally composed of detoxified PT and may also include FHA, pertactin and fimbriae (Fim2 and Fim3), in various combinations. FA is still the most widely used detoxifying agent of PT for use in acellular vaccines, but some manufacturers employ hydrogen peroxide, tetranitromethane or glutaraldehyde.

One company, Chiron-Biocrine, has created genetically detoxified PT as a component for their acellular vaccine (Rappuoli, 1996). They started with a cloned and sequenced 3.5-Kb DNA fragment, which contained the sequence for subunits S1 – S5 (Locht and Keith,

1986; Locht *et al.*, 1986; Nicosia *et al.*, 1986). To identify amino acids essential to enzymatic activity, a series of mutants was created, in the region of the S1 subunit containing amino acids 2 – 180, on the basis of sequence comparison with other bacterial toxins and computer modelling (Barbieri and Cortina, 1988; Burnette *et al.*, 1988; Locht *et al.*, 1990; Pizza *et al.*, 1988). The most successful mutant, and the one they use in their vaccine today, has two amino acid substitutions in the S1 catalytic site, Arg-9 → Lys and Glu-129 → Gly (PT-9K/129G) (Nencioni *et al.*, 1990). This antigen lacks ADP-ribosylation activity and other toxic effects of PT such as lymphocytosis and histamine sensitising activity. The antigen PT-9K/129G is also treated with FA, but at a much lower concentration (0.035 %, w/v) than required for detoxification, and for only 48 hours, in order to stabilise the protein (Nencioni *et al.*, 1991; Petre *et al.*, 1996).

Various clinical trials were carried out in the early 1990s in the USA, Italy, Germany, Sweden and Senegal, sponsored by The National Institute for Allergy and Infectious Diseases to compare the safety and immunogenicity of experimental pertussis vaccines (Klein, 1995; Decker *et al.*, 1995; Edwards *et al.*, 1995). In addition, manufacturers also conducted independent trials (Annunziato *et al.*, 1994; Marcinak *et al.*, 1993; Pichichero *et al.*, 1994; Halperin *et al.*, 1994). Although there were differences in the study designs between the different trials, some general comparisons and conclusions can be made (Hewlett and Cherry.J.D., 1997). The results showed that the acellular vaccines always produced fewer adverse reactions than the whole-cell vaccine. There was variation between the efficacy of the acellular vaccines (59 – 96 %), with most performing as well as or better than, and some performing worse than the whole cell vaccines. In general, the

efficacy of the acellular vaccines improved with an increasing number of antigen components. When the quantities of antigen in the acellular vaccines were compared, those vaccines containing genetically detoxified PT were more immunogenic than the acellular vaccines containing chemically detoxified PT (Klein, 1995).

In the search for the most efficacious combination of vaccine components, research has provided increasing information on the immunogenic properties of pertussis antigens, and is ongoing. Most antigens, with the exception of toxoided PT, are not protective on their own. FHA has been shown to be a poor immunogen in mice (Zackrisson, Taranger, and Trollfors, 1990; Sato and Sato, 1985) and anti-FHA does not contribute to the therapeutic activity of pertussis vaccines in children (Granstrom *et al.*, 1991). Pertactin only provides partial protection from pulmonary *B. pertussis* infection in mice (Shahin *et al.*, 1990) and has been shown not to be responsible for protection by pertussis vaccines (Aoyama *et al.*, 1989; Sato, 1997). Fimbriae have been shown to give some protection in mice (Robinson *et al.*, 1989; Jones *et al.*, 1995), but are not required for the elimination of pertussis in children (Aoyama *et al.*, 1989; Sato, 1997). Adenylate cyclase in contrast has been shown to confer protection against pertussis infection in mice (Brézin *et al.*, 1987; Imaizumi *et al.*, 1985) but due to its toxicity has not been included in the already successful acellular vaccines. PT is highly immunogenic conferring 100 % protection against *B. pertussis* pulmonary infection and intracerebral challenge in mice and a high degree of immunity in humans (Sato *et al.*, 1980; Sato *et al.*, 1981; Oda *et al.*, 1984; Sato and Sato, 1985; Robbins *et al.*, 1993; Trollfors *et al.*, 1995; Schneerson *et al.*, 1996). Studies show that a

combination of PT and other less immunogenic pertussis antigens is more protective than PT alone (Mills *et al.*, 1998; Sato and Sato, 1984).

Although the whole cell vaccine is still successful in many parts of the world, the arrival of the acellular vaccine has increased the scope for controlling whooping cough. Earlier vaccination of infants, vaccination of adolescents and mothers, and the production of combination vaccines are all advantages of the less reactogenic acellular over the whole cell vaccine (Rappuoli, 1996).

1.4.3 Vaccine testing

The study of disease in a suitable animal model in which the disease effects correlate as closely as possible with those in humans is essential for effective standardising and controlling of vaccine products. Humans are the only known hosts of *B. pertussis*, therefore establishing a suitable animal model is an ongoing challenge. Primates are the only known animals to suffer the paroxysmal cough and mucous production, which are characteristic of the human disease (Rich *et al.*, 1936). Rats also produce a cough and accumulate serum in the lungs but do not secrete the mucous as primates do (Hornibrook and Ashburn, 1939) and rabbits become infected but do not develop any systemic disease effects. Mice do not exhibit the classical cough or mucous secretions, but the bacteria attach and colonise mice lungs in the same way, and produce many of the same biological effects as they do in humans (Sato *et al.*, 1980). The mouse has become the model of

choice for studying *B. pertussis* disease, and in the standardisation and testing of pertussis vaccines.

The disease that develops in mice following intranasal inoculation or aerosol challenge resembles the disease in humans in a number of respects, including manifestations of PT action such as lymphocytosis (Sato *et al.*, 1981), hyperinsulinemia (Pittman, Furman, and Wardlaw, 1980), and mitogenesis (Morse *et al.*, 1977; Kong and Morse, 1977). Infant mice, like infant humans, may have significant mortality from the disease while older mice, like older humans, have less severe disease and are less likely to die (Pittman, Furman, and Wardlaw, 1980). In addition, both human and mice survivors of a sublethal dose of *B. pertussis* often develop a chronic infection that lasts several weeks to several months (Dolby, Thow, and Standfast, 1961; Weiss *et al.*, 1984; Sato *et al.*, 1981).

Protection against mouse IC challenge is widely used to test the efficacy of whole cell pertussis vaccines since protection in this model has been shown to correlate with whole cell vaccine efficacy in humans (Medical Research Council, 1956). However, the results have been variable, and the mechanism of protection by this route is not fully understood (Cameron, 1988; Preston and Stanbridge, 1976). Moreover, this method has been shown not to be suitable for potency testing of acellular vaccines. This may be due to the fact that protective potencies determined by IC challenge appear to be dependent largely on the presence of active PT in the vaccines. This is believed to be due to the ability of PT, even in minute quantities, to increase the permeability of the blood-brain barrier, allowing entry of antigens, antibodies and immune cells into the brain (Robinson and

Irons, 1983). Therefore, a small amount of active PT in the acellular vaccine may cause it to appear highly protective by the Kendrick test, even if it is poorly immunogenic. It has been considered that this test is better for the testing of whole cell vaccines since amounts of active PT will generally correlate with the presence of co-ordinately expressed immunogenic factors (Robinson and Funnell, 1992).

Efforts were made to design new animal protection tests for pertussis vaccines which more closely imitated natural challenge with *B. pertussis*, and which correlated with human protection. Intranasal infection by *B. pertussis* was studied (Dolby, Thow, and Standfast, 1961), and the protection of mice from lung colonisation was shown to correlate with vaccine efficacy in humans (Standfast, 1958). The aerosol challenge model was investigated and found to be a suitable alternative to the use of the Kendrick test (Xing *et al.*, 1999; Canthaboo *et al.*, 2000). Respiratory infection tests, by both aerosol and intranasal challenge, are being used increasingly in pertussis research, as alternatives to the Kendrick test.

Serum antibody responses are also analysed in vaccine testing. Although clinical trials have shown that levels of antibody do not always correlate with protection (Ad Hoc Group for the Study of Pertussis Vaccines, 1988; Tran Minh *et al.*, 1999), there is evidence that individuals without antitoxin are susceptible to whooping cough (Zackrisson, Taranger, and Trollfors, 1990; Isacson *et al.*, 1994). Vaccine testing in mice includes analysis of antibody production and safety, as well as antigen evaluation, to test

consistency of manufacture (Arciniega *et al.*, 1998), and studies of the immunological mechanisms of *B. pertussis* infection in mice and humans are ongoing.

1.5 Pertussis toxin (PT)

During their studies, Bordet and Genou noted that *B. pertussis* organisms were non-invasive and that disease continued after bacterial clearance (Bordet and Gengou, 1906). On this basis they proposed that whooping cough was a toxin-mediated disease but failed to isolate a toxin that imitated the disease effects. Seventy years later, a toxin that had been characterised with respect to a variety of biological activities, was implicated as the main immunogenic and toxic determinant of whooping cough, and it was named pertussis toxin (PT) (Pittman, 1979).

1.5.1 Structure and function

PT (Figure 1.4) is the best characterised virulence factor of the whooping cough bacterium. It belongs to the A-B class of bacterial protein toxins, which includes the cholera toxin and Shiga toxin families, *Pseudomonas* exotoxin-A and diphtheria toxin, all possessing functionally distinct catalytic (A) and cell-binding (B) domains (Sixma *et al.*, 1991; Stein *et al.*, 1992; Allured *et al.*, 1986; Choe *et al.*, 1992). The B-domain of PT binds to the host cell-surface sugar ligand, which is believed to facilitate membrane

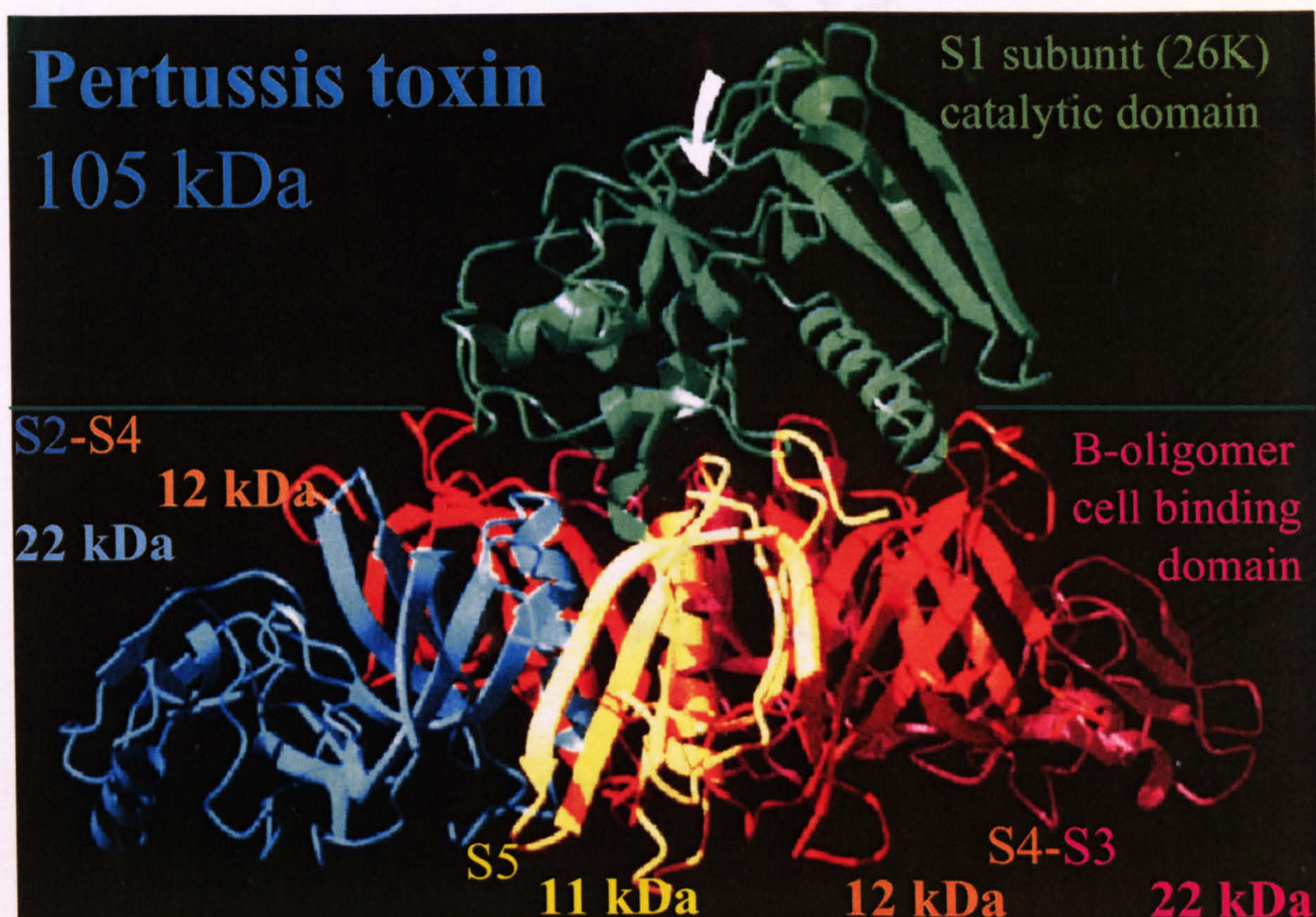


Figure 1.4 Pertussis toxin

The crystal structure of pertussis toxin, adapted from Stein, P. *et al.*, 1994a. Molecular weight labels match the subunit colours; the white arrow indicates the predicted NAD-binding cleft.

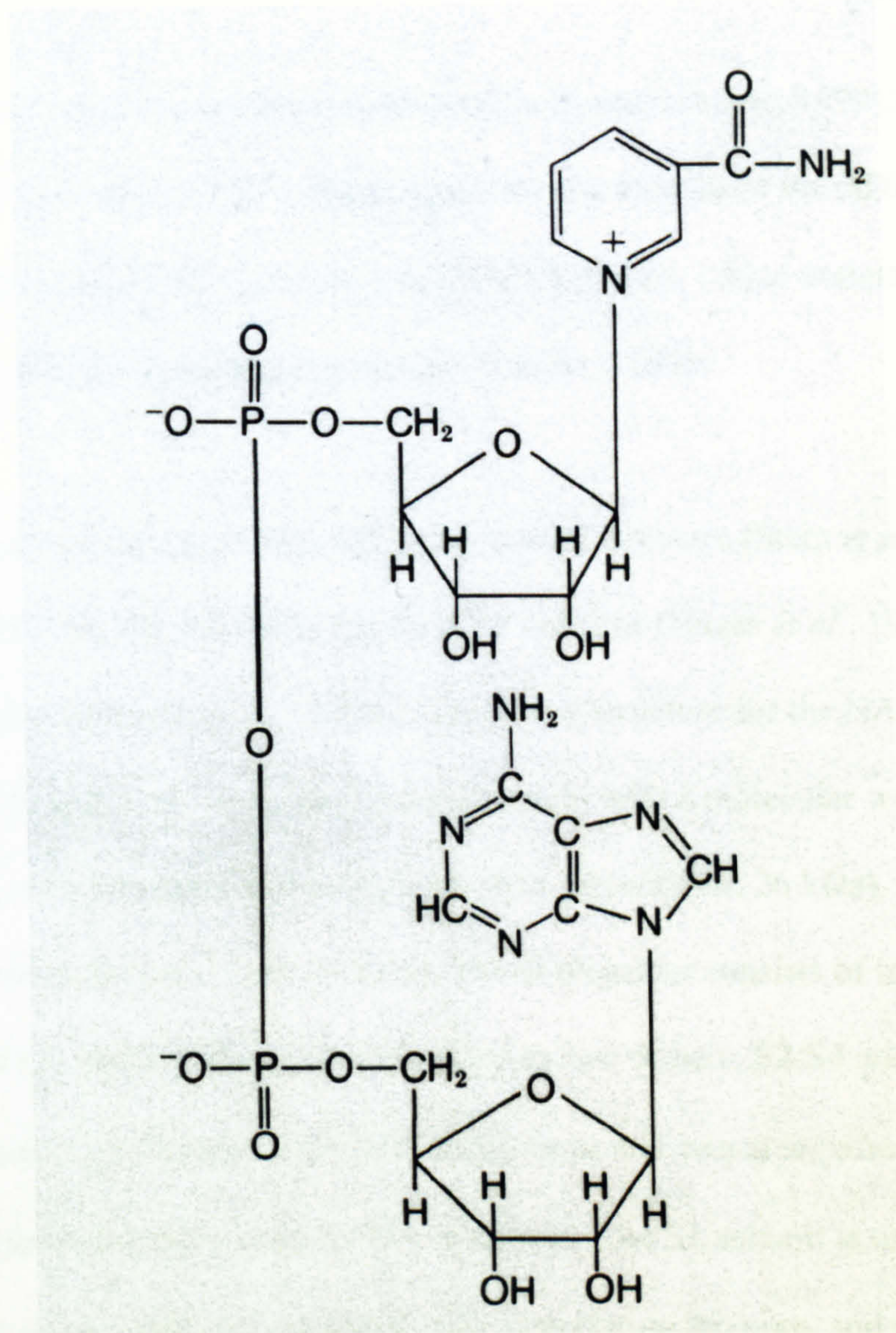


Figure 1.5 Structure of NAD⁺

translocation of the A-domain into the cytoplasm (Locht and Antoine, 1999). Following internalisation, the A-domain, or S1 subunit, catalyses the transfer of the ADP-ribosyl group from nicotinamide adenine dinucleotide (NAD^+) (Figure 1.5) to certain G-proteins, disrupting normal cellular signalling (Locht and Antoine, 1999).

The sequence (Locht and Keith, 1986) and X-ray crystal structure (Stein *et al.*, 1994a) of PT are known, including the structures for the ATP cofactor (Hazes *et al.*, 1996) and sugar ligand complexes (Stein *et al.*, 1994b). The X-ray structure for the NAD^+ substrate complex is yet to be resolved. PT is a hexameric protein with a molecular weight of 105 kDa (Figure 1.4), which comprises a catalytic A (S1) subunit (M_r , 26 kDa), and a cell-binding pentameric B-oligomer (M_r , 79 kDa). The B-oligomer consists of subunits S2, S3, two copies of S4, and S5. These are assembled as two dimers, S2-S4 and S3-S4, associated by extensive anti-parallel β -sheet interactions and joined together with S5 to form a central asymmetric pore lined by five α -helices. The S1 subunit is orientated on top of this ring structure with its C-terminal 'tail' penetrating the pore, and contacts in the A/B-domain interface are made by hydrogen bonds and other electrostatic interactions (Stein *et al.*, 1994a).

The subunits of PT can be resolved on an SDS-PAGE gel into four bands with S4 and S5 co-migrating (Sekura and Zhang, 1985). The subunits cannot be resolved using size exclusion chromatography even in the presence of guanidine hydrochloride. In these conditions a broad main peak and a small trailing shoulder is obtained from which elution

fractions contain mainly S1 at the start of elution, then S1/S2/S3 and lastly S4/5 (Sekura and Zhang, 1985).

The protein has a high β -sheet, low α -helix content, calculated to be 16 % α -helix and 37 % β -sheet, 11 % turns and 35 % other secondary structure, from the x-ray crystal structure (Appendix 1); and 10 % α -helix, 53 % β -sheet and 37 % β -turn/loop secondary structure from analysis of the protein in solution using circular dichroism (Seabrook, Atkinson, and Irons, 1991). It contains six tryptophan residues distributed between the subunits (S1 W26, W215; S2 W52, W143; S3 W52; S5 W52), which makes intrinsic fluorescence spectroscopy a valuable tool of PT tertiary structural analysis.

S1 subunit

In host cells, the S1 subunit of PT modifies certain inner cell membrane proteins, known as guanyl nucleotide-binding proteins (G-proteins). G-proteins translocate cell receptor signals, commonly to adenylate cyclase as well as other signal mediator proteins. The S1 subunit catalyses the ADP-ribosyl transfer from NAD^+ to a cysteine residue of the inhibitory α -subunit ($i\alpha$) of heterotrimeric G-proteins. This uncouples the $G_{i\alpha}$ subunit from its receptor, which leads to permanent activation of adenylate cyclase to produce the second messenger cAMP, and thus activation rather than inhibition of the receptor mediated signal (Ui, 1990).

The active site of the S1 subunit is in the form of a cleft, which appears to be a structural motif common to other ADP-ribosylating toxins such as *Escherichia coli* heat-labile enterotoxins (LT1 and LT2), cholera toxin (CT), *Pseudomonas aeruginosa* exotoxin-A

(PAETA) and diphtheria toxin (DT) (Stein *et al.*, 1994a; Sixma *et al.*, 1991; Sixma *et al.*, 1993; Allured *et al.*, 1986; Choe *et al.*, 1992). Conserved amino acids have been located at identical positions in the three-dimensional structures of these toxins, which have been shown through site-directed mutagenesis to be essential for ADP-ribosylating activity (Domenighini *et al.*, 1994; Pizza *et al.*, 1988). The motif is composed of an α -helix, β -strand and a large loop creating a partially open hydrophobic cavity. In DT, this pocket is the binding site for NAD^+ (Bell and Eisenberg, 1996) and has therefore been postulated as a structurally conserved NAD^+ binding site. The dissociation constant (K_d) for the PT- NAD^+ interaction was determined to be 24 μM at 30 $^\circ\text{C}$, by measuring quenching of PT fluorescence by NAD^+ (Seabrook, Atkinson, and Irons, 1991).

The enzymatic mechanism has been studied by transition-state modelling by studying kinetic isotope effects of the ADP-ribosylation reaction of PT with the $\text{G}_{i\alpha 1}$ subunit, in the presence of NAD^+ (Figure 1.5) (Scheuring, Berti, and Schramm, 1998). The results suggest a mechanism involving PT-induced cleavage of the C-N bond between the anomeric carbon of ribose and the nicotinamide nitrogen, and transfer of the ADP-ribosyl group to an ionised sulphydryl of the $\text{G}_{i\alpha 1}$ subunit cysteine residue.

The ADP-ribosyl transferase activity of the S1 subunit of PT is fully activated *in vitro* by chemical reduction (Moss *et al.*, 1986; Moss *et al.*, 1983). Observation of its three dimensional structure has lead to the suggestion that effective NAD^+ binding in the catalytic site requires cleavage of the Cys-41 – Cys-201 disulphide bond to allow

displacement of the α -helix (amino acids 184 – 203), which appears to occlude the NAD⁺ binding site (Stein *et al.*, 1994a; Bell and Eisenberg, 1996).

B-oligomer

The B-oligomer is required for entry of the toxin into the host tracheal epithelial cells.

The B-oligomer binds by at least two binding sites, located on its S2 and S3 subunits (Stein *et al.*, 1994b). Studies have shown that the most likely host cell ligands are oligosaccharides with terminal sialyllactosamine residues on glycolipids or N-linked glycoproteins (Witvliet *et al.*, 1989; Brennan *et al.*, 1988; Sandros *et al.*, 1994).

Binding to the host cell surface is believed to initiate endocytosis of the holotoxin.

Experiments have implicated that the toxin is trafficked through the Golgi and endoplasmic reticulum (Xu and Barbieri, 1995). However, the sequence of events leading to targetting of the A subunit to its substrate is not fully understood.

It is known that the A subunit must be separated from the B-oligomer for catalytic activity and that dissociation of the domains is likely to be promoted by ATP binding to the B-oligomer (Hazes *et al.*, 1996; Moss *et al.*, 1986). ATP-binding is believed to destabilise the interaction between the B-oligomer and A subunit. However, it is not known where in the host cell this happens.

1.5.2 Biological activities

PT is a unique protein possessing many important biological properties most of which were described before the purification and characterisation of the toxin. Accumulation of early evidence indicated that these properties were the actions of one protein (Munoz, 1985). The mechanisms of the varied effects of PT are now beginning to be appreciated and are still the subject of intense study. It is now known that many of the diverse actions of PT, observed *in vivo* and in *in vitro* cell cultures such as CHO-cells, are dependent ultimately on the catalytic activity of the protein, and some are dependent on the binding properties of the B-oligomer alone. PT-modified G-proteins are unable to regulate normal homeostatic inhibition of signal-mediating proteins, such as adenylate cyclase. PT-induced effects, on adenylate cyclase-mediated pathways, include histamine sensitisation and insulin secretion. G-protein modification can also affect other signal-mediating proteins (Munoz, 1985). Another important biological effect of PT is its adjuvanticity, in which the B-oligomer and catalytic activity may have defined roles. In contrast, the haemagglutinating and mitogenic activities of PT are dependent on B-oligomer binding alone (Nencioni *et al.*, 1991).

Chinese hamster ovary (CHO)-cell toxicity

Numerous effects of PT on cells *in vitro* have been described, including the enhancement of insulin release from rat pancreatic islet B-cells, the enhancement of agonist-induced rat C6 glioma cell activity and rat heart cell activity and toxicity to CHO-cells (Munoz, 1985). These activities are all dependent on both translocation of the toxin into the cell

and catalytic modification of G-proteins, thus on both the A- and the B-domain. CHO-cells are used in many laboratories as model cell lines to study the cellular exploits of PT.

CHO-cells bear a 165-kDa glycoprotein on their surface that is recognised by PT B-oligomer (Brennan *et al.*, 1988), and contain a 41-kDa inner-membrane protein that is ADP-ribosylated by PT S1 subunit (Burns, Kenimer, and Manclark, 1987).

Unaffected CHO-cells can be observed under a light microscope as elongated cells in a uniform monolayer. Culture in the presence of as little as 30 pg of PT per 5000 cells, causes a characteristic clustering effect and change in the cell morphology, to a more square shortened cell, 16 hours following the addition of PT (Hewlett *et al.*, 1983; Gillenius *et al.*, 1985). The toxic effect of PT on CHO-cells has been employed in the development of assays to test the toxicity of PT-based vaccines and the PT neutralising capacity of anti-PT serum (Gillenius *et al.*, 1985). It is an accepted alternative to the animal toxicity test (histamine-sensitising test) as described in the European Pharmacopoeia (European Pharmacopoeia, 2001).

Histamine-sensitising activity

One of the first recognised biological activities of PT was its histamine sensitising effects for which it was known as histamine sensitising factor. Early experiments showed that mice immunised with the pertussis vaccine had increased susceptibility to the effects of histamine (Parfentjev and Goodline, 1948; Clausen, Munoz, and Bergman, 1968; Munoz and Bergman, 1968). Histamine causes vasodilation, promotes capillary permeability and

bronchoconstriction and can cause rapid death in mice previously immunised with active PT. Sensitivity to histamine occurs about 90 minutes after inoculation with PT and is long lasting, being detected months later. Histamine sensitisation is now known to be dependent on the ADP-ribosylating activity of PT (Nencioni *et al.*, 1991). However, the mechanism of PT-induced hypersensitivity to histamine is not clear but is thought to be due in part to PT mediating its effects via the histamine receptors H1, H2 and H3, or via pathways controlled by histamine receptors such as the nitric oxide synthase system (Vleeming *et al.*, 2000; Nilsson, Kockum, and Rosengren, 1997). Laboratories test the toxicity of pertussis vaccines and PT preparations by challenging mice with histamine after immunisation. Toxic vaccines or PT preparations cause anaphylactic shock and death of the mice within minutes of the challenge.

Leukocytosis promoting activity

Leukocytosis promoting factor was another agent subsequently revealed as PT. This activity was first observed in children infected with *B. pertussis* (Fröhlich, 1897) and since then in experimental animals receiving the pertussis vaccine or PT (Munoz and Bergman, 1977). Leukocytosis, an increase in the number of circulating leukocytes, is evident one day after injection of PT, reaches a maximum after about four days and then gradually declines to normal levels. Leukocytosis is predominantly due to an increase in lymphocytes including B- and T-cells, but other white blood cells such as polymorphonuclear leukocytes are also increased (Morse and Morse, 1976). Leukocytosis is followed by a decrease in the weight of the thymus and lymph nodes and an increase in spleen weight (Munoz, 1985). Even after extensive *in vitro* cultivation in the absence of

PT, the PT-treated leukocytes retained their inability to migrate into lymph nodes *in vivo* (Spangrude, Braaten, and Daynes, 1984). It has been concluded that leukocytosis is not due to multiplication of leukocytes, but to the prevention of their re-circulation through the lymphoid tissue once they have been released (Morse, 1976; Spangrude, Braaten, and Daynes, 1984). Leukocyte promoting activity is dependent on the ADP-ribosylating activity of PT (Nencioni *et al.*, 1991), and it has been proposed that the mechanism of PT-induced leukocytosis is dependent on adenylate cyclase-activated events (Braaten, Spangrude, and Daynes, 1984). Counting lymphocyte numbers in the whole blood of immunised mice is used as a check for pertussis vaccine toxicity.

Other activities

A whole host of other effects of PT have been observed, the most noteworthy of which include its islet activating effect, potent adjuvant activity, mitogenic and haemagglutination activities.

PT possesses the ability to activate pancreatic islets to produce insulin and thus was previously known as islet activating factor. This was initially observed as the attenuation of catecholamine inhibition of insulin release in rats, which had been previously immunised with pertussis vaccine (Munoz, 1985). Subsequent studies revealed that PT, via G-protein modification, attenuates the inhibitory effects of a variety of other hormones (Ui, 1988).

Greenberg and Fleming first observed the adjuvant activity of the pertussis vaccine in 1947 (Greenberg and Fleming, 1947), when they noted that tetanus and diphtheria toxoids given to children in combination with pertussis vaccine induced a higher production of antitoxins than when the toxoids were given alone. The adjuvant properties of the vaccine are now known to be due to the actions of lipid A of LPS (Ayme *et al.*, 1980) and to PT (Munoz and Bergman, 1977). Various groups have studied the nature of the immune response and mechanism of PT action in adjuvanticity. The current literature implies a role for both the A- and B- domains in this activity. The adjuvant action of PT will be discussed in more detail later in this chapter (section 1.5.3).

Other activities of PT are the T-cell mitogenic and haemagglutination activities which, in contrast to the above, are conferred by the B-oligomer alone (Nencioni *et al.*, 1990). Separate B-oligomer dimers (i.e. S2 – S4, S3 – S4) cannot induce mitogenicity therefore it has been suggested that cross-linking of cell-surface proteins causes mitogenic activity (Tamura *et al.*, 1983). In contrast to mitogenic activity, haemagglutination by the S2-S4 dimer is as effective as by the whole B-oligomer (Irons and MacLennan, 1979) (Nogimori *et al.*, 1985), suggesting different B-oligomer binding sites for the erythrocytes than for T-cells.

1.5.3 Immunological characteristics

PT has proved to be the most protective of the *B. pertussis* antigens alone in mice, and some believe PT alone to be adequate to compose a protective acellular pertussis vaccine

(Robbins *et al.*, 1993). Both humoral and cellular responses, specific for PT, appear to play a key role in clearing *B. pertussis* infection. One of the most intriguing aspects of PT is that it can induce a heightened immune response to coinjected antigens either from *B. pertussis* or other unrelated antigens, although the mechanism of the adjuvant action of PT has still to be elucidated. Many protective T- and B-cell epitopes have been identified in the PT molecule.

Immunogenicity

PT is a potent immunogen. Passive immunisation with antibodies specific for PT, has been shown to have a therapeutic effect in both mice (Sato *et al.*, 1981) and children (Granstrom *et al.*, 1991) infected with *B. pertussis*. PT, in active immunisation, can also confer high levels of protection against murine pulmonary infection or intra-cerebral challenge with *B. pertussis* (Oda *et al.*, 1984; Sato and Sato, 1985). Moreover, a monocomponent PT vaccine has been shown to confer good protection in humans (Trollfors *et al.*, 1995).

The clearance of *B. pertussis* infection is dependent on humoral (mediated by Th2 cells) and cell-mediated immunity, (mediated by Th1 cells) (Mills *et al.*, 1998) directed against PT as well as other *B. pertussis* antigens (Thomas *et al.*, 1989; Tran Minh *et al.*, 1999). Antibody responses, especially against PT, have long been regarded as important in fighting *B. pertussis* infection (Sato *et al.*, 1981; Granstrom *et al.*, 1991; Thomas *et al.*, 1989). Further evidence from studies using transgenic mice, have shown that B-cells or their antibody products are essential for the clearance of *B. pertussis* from the lungs

(Mahon *et al.*, 1997; Mills *et al.*, 1998). However, a direct correlation between serum antibody titres and protection from disease has not been established (Duchen *et al.*, 1997; Ad Hoc Group for the Study of Pertussis Vaccines, 1988; Tran Minh *et al.*, 1999). It has been shown in mice and humans that acellular vaccines tend to preferentially induce the production of IL-4 and IL-5 cytokines and antibodies, typical of a Th2 response (Ausiello *et al.*, 1997; Hall, Parton, and Wardlaw, 1998; Redhead *et al.*, 1993; Nilsson *et al.*, 1998) but depending on the preparation they can induce a mixed Th1/Th2 response (Ausiello *et al.*, 1997; Ryan *et al.*, 1997a; Ryan *et al.*, 1998a; Di Tommaso *et al.*, 1997).

Cellular immunity is more recently being recognised as an important mechanism of protection, although it has been known for some time that macrophage activity is associated with *B. pertussis* infection (Cheers and Gray, 1969) and that *B. pertussis* can survive within human macrophages (Cheers and Gray, 1969; Masure, 1993; Bromberg, Tannis, and Steiner, 1991; Ewanowich *et al.*, 1989). Experiments have shown correlation between protection and various cell-mediated responses. One study reported that the least severe clinical symptoms of school children infected with *B. pertussis* correlated with good *in vitro* proliferative responses of their peripheral blood mononuclear cells when induced by PT, FHA and/or pertactin (Tran Minh *et al.*, 1999). Also, the induction of Th1-mediated cellular immune responses such as interferon (IFN)-gamma and interleukin (IL)-2 has been shown to be essential for protection against *B. pertussis* infection (Redhead *et al.*, 1993; Mahon *et al.*, 1997). Infection with *B. pertussis* or inoculation with the whole cell vaccine has been shown to preferentially induce these Th1 type responses (Ryan *et al.*, 1997b; Ausiello *et al.*, 1997; Mahon *et al.*, 1996).

Adjuvant activity

PT is not only a protective immunogen, but also has potent adjuvant activity. This phenomenon was originally observed as the enhancement of local and systemic antibody responses, including IgE, IgA and IgG, to antigens co-administered with native PT (Greenberg and Fleming, 1947; Munoz and Bergman, 1977; Munoz *et al.*, 1981; Munoz and Peacock, 1990; Roberts *et al.*, 1995). Native PT has also been shown to enhance protection against IC and aerosol challenge with *B. pertussis* when co-administered in small amounts with other *B. pertussis* antigens (Robinson and Irons, 1983; Xing, Canthaboo, and Corbel, 2000). Furthermore, this protection has been shown to correlate with an increase in nitric oxide production by macrophages, an indication of macrophage activation (Xing, Canthaboo, and Corbel, 2000). In addition, it has been demonstrated that PT potentiates both Th1 and Th2 murine responses to co-injected antigen by causing an increase in IL-2, IFN-gamma, IL-5, IL-4, IgG1 and IgG2a production and in the proliferative response of spleen cells (Ryan *et al.*, 1998b).

The mechanism of the immunopotentiating action of PT is not yet fully understood, but the available data suggest that the A and B-domains have distinct effects. In one experiment, both native PT and catalytically inactive mutant PT (5 µg/dose) were shown to have adjuvant activity, as demonstrated by the enhanced stimulation of Th1 and Th2 cytokines in mice (Ryan *et al.*, 1998b). Two other studies in mice showed that a much smaller amount of native PT (0.12 or 0.2 µg/dose) stimulated an enhanced cytokine response (Shive *et al.*, 2000), nitric oxide production by macrophages, and protection

(Xing, Canthaboo, and Corbel, 2000), but that the same quantity of the isolated B-oligomer, or mutant PT, caused considerably less enhancement.

Immune epitopes

Many studies of the T- and B-cell epitopes of PT have been carried out. Details of the PT subunit amino acid sequences that have been identified as T- or B-cell antigenic regions are presented (Table 1.1).

Protective B-cell epitopes are abundant on the PT molecule, exemplified by the abundant protective PT subunit-specific mAbs produced by Hiroko Sato and co-workers. They have identified a total of 32 mAbs specific for epitopes on PT subunits S1 – S5. (Sato, Sato, and Ohishi, 1991; Sato *et al.*, 1984). Of these, the 1B7 mAb specific for an epitope on the S1 subunit, contained within residues 8 – 15 (Table 1.1), has proved to be the most protective (Cieplak *et al.*, 1988; Sato, Sato, and Ohishi, 1991; Sato *et al.*, 1984; Sato and Sato, 1990). Some B-cell epitopes may be discontinuous or conformational, or both, and therefore difficult to map by mAb screening of linear peptides, as was found for epitopes of the S4 subunit (Ibsen *et al.*, 1993).

Human T-cell antigenic regions of S1 and S4, and mouse T-cell antigenic regions of S3 and S4 have also been identified, by studying T-cell proliferative responses to synthetic peptides or recombinant subunit fragments (Table 1.1). Within the human T-cell

Table 1.1 Antigenic sequences of PT

A summary of published studies, identifying T- and B-cell antigenic sites on the pertussis toxin (PT) molecule.

PT Subunit	Amino acid sequence	Epitope type	Experimental details	Reference
S1	8-15	1B7 mouse antibody binding region	First 30 amino acids progressively truncated; blots probed with 1B7	(Sato <i>et al.</i> , 1984a)
S1	1-42 181-211 212-235	T-cell antigenic regions (human)	T-cell proliferation using a series of recombinant truncated fragments; computer analysis	(de Magistris <i>et al.</i> , 1989; Peppoloni <i>et al.</i> , 1995)
S1	Leu33 and Leu36 Asp34, His35, Thr37 and Arg39	DR1 binding T-cell receptor recognition (human)	Peptide containing amino acids 30-42 and alanine-substituted analogues screened for DR1 binding and T-cell proliferation	(de Magistris <i>et al.</i> , 1992)
S3	18-41 78-108 134-154 149-176	T-cell antigenic regions (mouse)	Synthetic peptides induced T-cell proliferation	(Chong <i>et al.</i> , 1992)
S4	1-15 21-45 41-65	T-cell antigenic sites (human)	Proliferation assays using synthetic peptides	(Petersen <i>et al.</i> , 1992)
S4	21-45 72-88 93-106	T-cell antigenic sites (mouse)	Proliferation assays using synthetic peptides	(Petersen <i>et al.</i> , 1993)

antigenic region encompassing residues 1 – 42 of the S1 subunit (de Magistris *et al.*, 1989), specific residues required for interaction with the major histocompatibility (MHC) molecule, DR1, (Leu-33 and Leu-36) and for specific T-cell receptor recognition (Asp-34, His-35, Thr-37 and Arg-39) have been identified using alanine-substituted analogues of subunit peptides (de Magistris *et al.*, 1992). In support of these results, computer algorithms designed to identify T-cell binding determinants, selected S1 residues 31 – 34 (de Magistris *et al.*, 1992) as a T-cell-binding consensus motif (Rothbard and Taylor, 1988). Furthermore, S1 residues 26 – 42 were predicted to form an amphipathic helix common to T-cell binding sequences (Margalit *et al.*, 1987). On the B-oligomer, particularly noteworthy is the epitope on the S4 subunit (residues 21 – 45) which was identified as a T-cell antigenic region in both mice and humans (Petersen *et al.*, 1993; Petersen *et al.*, 1992).

Antigenic variation of PT

It has identified that specific amino acid differences between known S1 variants, A (Asp-34) and D (Glu-34) and between A (Ile-194) and B (Met-194), lie within T-cell epitopes in the S1 subunit (Mooi *et al.*, 1998). It has been speculated that such antigenic differences between vaccine strains of *B. pertussis* and those circulating in the population may make for a less effective pertussis vaccine, and that this was partly the cause of the re-emergence of pertussis in The Netherlands (Mooi *et al.*, 1998). This group has postulated that it may be important for the efficacy of acellular vaccines, which are based on a few purified antigens, to ensure the antigen type in the vaccine matches the circulating antigen type. However, this issue is yet unresolved.

1.6 Effect of toxoiding on PT

1.6.1 Genetic detoxification

Unlike wild-type PT, the genetically detoxified PT (PT-9K/129G) used in the Chiron-Biocrine acellular pertussis vaccine, is devoid of ADP-ribosyl transferase activity. Thus, PT-9K/129G cannot induce CHO-cell toxicity, leukocytosis, hyperinsulinemia or histamine sensitivity (Nencioni *et al.*, 1990). However, PT-9K/129G has an SDS-PAGE pattern indistinguishable from wild-type PT (Nencioni *et al.*, 1990). Also comparable with wild-type PT, are its T-cell mitogenic activity, haemagglutinating activity and affinity for PT-specific polyclonal antibodies and the protective monoclonal antibody (mAb) 1B7 (Nencioni *et al.*, 1990). PT-9K/129G can also protect mice in a dose-dependent fashion against IC challenge with *B. pertussis* (Nencioni *et al.*, 1990). Chiron-Biocrine have reported that they stabilise the mutant PT with 0.035 % (w/v) FA (Rappuoli, 1994).

1.6.2 Chemical detoxification

A variety of chemicals, notably glutaraldehyde, tetranitromethane, hydrogen peroxide, and FA are used by different manufacturers to toxoid the PT component of their DTP vaccine (Edwards *et al.*, 1995; Siber *et al.*, 1991). With the exception of FA, there have been few studies on the effects of these chemicals on PT, and to date they have focused

on PT-specific murine antibody responses, and mAb binding to PT epitopes. One study showed that DPT vaccines containing PT detoxified with glutaraldehyde, tetranitromethane or FA, or containing PT-9K/129G stabilised with 0.035 % FA, induced good PT-specific antibody responses in mice, relative to the DTP vaccine containing the whole cell pertussis component. The order of this antibody response to the different DTP vaccines (referred to by PT toxoid), from highest to lowest was: FA-stabilised PT-9K/129G, glutaraldehyde-treated PT, whole cell *B. pertussis*, FA-treated PT, tetranitromethane-treated PT (Edwards *et al.*, 1995). Another study has shown that hydrogen peroxide and FA cause a reduction in PT epitopes, as determined by PT-neutralising murine mAb binding (Ibsen, 1996). It has also been demonstrated that glutaraldehyde treatment reduces the toxicity of crude PT preparations more effectively than FA, as determined by histamine sensitisation, CHO-cell toxicity assay and IC challenge, (Gupta *et al.*, 1990; Gupta *et al.*, 1991). The effect of glutaraldehyde on the neutralising capacity of anti-PT serum was also found to be less damaging than FA (Gupta *et al.*, 1991). The effects of these chemicals on the structural properties of PT and a full comparison of antigenic and protective properties of the non-combined PT toxoids have not yet been investigated.

1.6.3 FA-treatment

The effects of FA on properties of PT have been more extensively analysed than the effects of the other toxoiding chemicals. In addition, the chemistry of FA reactions with amino acids has been studied (Tome and Naulet, 1981; Tome, Kozlowski, and Mabon,

1985; Walker, 1964; Feeney, Blankenhorn, and Dixon, 1975; Means and Feeney, 1968). FA was employed as the PT toxoiding agent in the first acellular pertussis vaccine developed in Japan, and is still used world-wide to toxoid PT and in lower concentrations to stabilise PT-9K/129G (Edwards *et al.*, 1995; Sato, Kimura, and Fukumi, 1984; Nencioni *et al.*, 1991). The Japanese DTP vaccine containing the PT toxoid was tested for residual biological activities of PT using leukocyte proliferation and histamine sensitisation tests (Sato, Kimura, and Fukumi, 1984). More direct studies were performed using a partially purified native PT preparation to assess the effect of FA on PT toxic properties and anti-PT neutralising antibodies (Gupta *et al.*, 1991). Studies on the effects of FA on PT-9K/129G have involved more detailed analysis on biological and immunological properties of the antigen (Nencioni *et al.*, 1991). Preliminary structural studies on the effects of FA on PT-9K/129G and native PT have also been undertaken (Nencioni *et al.*, 1991; Bolgiano *et al.*, 1999).

1.6.3.1 Chemistry of FA reaction with amino acids

FA reacts with groups containing a reactive hydrogen atom (nucleophile) to form a reactive hydroxymethyl compound, which may undergo a condensation reaction with another nucleophile to produce a methylene bridge (Walker, 1964; Tome and Naulet, 1981). In proteins, lysine is one of the most reactive amino acids with FA (Tome, Kozlowski, and Mabon, 1985). The first stage in the reaction is the hydroxymethylation of the ϵ -amino group of lysine, which is rapid and reversible (Feeney, Blankenhorn, and

Dixon, 1975; Means and Feeney, 1968) and then a slow condensation reaction proceeds with another amino acid side-chain to form a methylene bridge (Tome, Kozlowski, and Mabon, 1985). Methylene bridges and other FA-induced amino acid modifications have been detected in FA-treated bovine serum albumin using NMR spectroscopy (Tome, Kozlowski, and Mabon, 1985).

Lysine is added to the reaction mixture during FA-treatment of PT and PT-9K/129G, for the production of vaccine toxoids, and in the production of experimental toxoids in this study. In the absence of added lysine, extensive denaturation, aggregation and precipitation occurs presumably due to extensive inter- and intra-molecular bridging (Petre *et al.*, 1996). The amino acid additives serve to quench the massive denaturing effects of FA by creating active lysine species, which can then specifically react with amino acid side chains in a slower controlled reaction. The reaction of FA with free lysine most readily occurs with lysine at the ϵ -amino group of the side-chain, since the α -amino group is usually unreactive under the conditions of the reaction (Tome and Naulet, 1981).

1.6.3.2 Biological and Immunological properties of FA-treated PT

FA-treated native PT antigen was developed as a component of the first acellular vaccine in Japan, in the early 1980's (Sato, Kimura, and Fukumi, 1984), and is still used in current acellular vaccines. In the Japanese acellular vaccine PT is treated with increasing

amounts of FA every 3 days to a final concentration of 0.4 % (w/v) FA at 37 °C and incubated further for an unspecified number of days (Sato, Kimura, and Fukumi, 1984). However, the effect of FA on PT has not been examined. The FA-treated PT component has not been examined individually, but the DTP vaccine containing the acellular pertussis component was compared with the DTP vaccine containing whole-cell pertussis. The DTP vaccine containing the acellular pertussis component was shown to be as protective as the conventional Japanese whole cell vaccine as determined by IC challenge, and had one-tenth of the toxicity of the whole cell vaccine as determined by mouse-body-weight-decreasing activity, leucocyte-promoting activity and histamine-sensitising assays. The Japanese PT toxoid has been used to immunise mice, for the production of hybridomas to generate mAbs. Some of these mAbs were found to recognise native PT epitopes. They also protected mice against *B. pertussis* by both IC and aerosol challenge (Sato *et al.*, 1984; Sato and Sato, 1984; Sato and Sato, 1990; Sato *et al.*, 1987; Sato, Sato, and Ohishi, 1991).

Another group analysed the effect of FA on partially purified PT preparations treated with up to 0.2 % FA, at 37 °C for 24 hours (Gupta *et al.*, 1991). These samples exhibited a decrease in CHO-cell toxicity, leukocytosis activity and histamine sensitising activity. However, residual activity was still present even with 0.2 % FA-treatment and the preparations did not pass the mouse weight gain test. These preparations also demonstrated a decrease in the induction of PT neutralising antibodies with FA-treatment. Studies on the FA-treatment of PT-9K/129G were more detailed and focused on specific changes in antigenic properties. The effects of FA on the genetic mutant PT-

9K/129G have been investigated, in view of using FA as a stabilising agent, since without FA treatment this antigen and other purified *B. pertussis* antigens were unstable and subject to degradation (Petre *et al.*, 1996).

PT-9K/129G is devoid of toxic biological properties, dependent on the S1 catalytic activity, but retains biological activities associated with the B-oligomer as well as protective and antigenic properties. Studies were carried out on PT-9K/129G, which had been treated with various concentrations of FA, up to 0.42 % (w/v), at 37 °C for 48 hours. At low concentrations of FA, up to about 0.05 % (w/v), mitogenic and haemagglutinating activities, as well as PT-specific polyclonal antibody affinity, were comparable with untreated PT-9K/129G (Nencioni *et al.*, 1991). With the highest FA-treatment, mitogenic and haemagglutinating activities were almost totally eliminated and antibody-binding affinities declined, although recognition of PT-9K/129G by human T-cell clones was not affected. Another study showed that much larger concentrations of FA, 3.5 % FA (w/v), constrained the presentation of PT-9K/129G T-cell epitopes on S4, and promoted the presentation of epitopes on S1, S2 and S3 (Di Tommaso *et al.*, 1994).

1.6.3.3 Physico-chemical properties of FA-treated PT

There is evidence that commercial PT toxoids, including FA-treated PT9K/129G and FA-treated native PT, contain higher molecular weight species than untreated PT. These toxoids displayed the typical SDS-PAGE band pattern of untreated PT, but with

additional higher molecular weight bands (Bolgiano *et al.*, 1999; Nencioni *et al.*, 1991). Moreover, in Western blotting the S1 subunit of PT-9K/129G appeared in a higher molecular weight band after FA treatment. It was also shown to produce earlier eluting species in size exclusion chromatography (SEC) (Ceccarini *et al.*, 2000). In addition, the commercially toxoided native PT produced a modified intrinsic fluorescence spectroscopy spectrum (Bolgiano *et al.*, 1999). The structural nature of these different forms of PT produced by FA treatment has not been explored nor their sizes confirmed, and the effect of FA on PT conformation has not been clarified.

There is little understanding of the effect of the different toxoiding procedures on secondary, tertiary and quaternary protein structure. Furthermore, no attempt has yet been made to associate any of the molecular forms of PT, which may arise with toxoiding, with antigenicity or immunological responses. A thorough understanding of the molecular forms produced from toxoiding is a necessary prelude to determining the roles of these molecules forms in directing the immune response. In addition, guidelines by the World Health Organisation have emphasised the importance of physicochemical in addition to immunogenicity testing for the standardisation of pertussis vaccines (Arciniega *et al.*, 1998). The advantages of establishing molecular correlates of immunogenicity or protection also extend to the development of *in vitro* assays as alternatives to the use of animals. Determining how the physico-chemical nature of PT toxoids relate to their antigenic and protective properties will drive the development of better-defined and potentially more immunogenic pertussis vaccines.

1.7 Thesis format

Following the general introduction (Chapter 1) is the Materials and Methods section (Chapter 2) which describes the experiments of the entire thesis, and is divided up by experiment type. Five Results chapters follow this, each including a specific introduction and summation. The final chapter is an overall discussion including a general review of the data and a discussion of their implications in the context of the thesis.

Chapter 1 Introduction

The Introduction is a general assessment of the background work explaining the context and the overall aims of the thesis.

Chapter 2 Materials and Methods

This Chapter covers the materials and methods for all of the experiments described in the thesis.

Chapter 3 Effect of ‘activation’ and NAD⁺ binding on the structure of the S1 subunit of PT

The effect of ‘activation’ (by cleavage of disulphide bond Cys-41 – Cys-201) and NAD⁺ binding on the PT structure were studied using intrinsic fluorescence spectroscopy. Computer modelling of PT was carried out to generate an NAD⁺-bound model of PT and the results compared with those from fluorescence spectroscopy.

Chapter 4 Effect of chemical toxoiding on PT structure, epitopes and murine immune response

Different commercial PT toxoids, which include the FA-stabilised mutant PT and those which are produced by treatment with different chemicals, were analysed for structural changes using SDS-PAGE, size exclusion chromatography (SEC) and fluorescence spectroscopy. Immunological changes were analysed by immunoblotting and ELISA, using subunit-specific mAbs. In addition, PT-specific murine IgG antibodies raised against each PT preparation were quantified and the protective efficacy of each preparation was also investigated using an aerosol challenge assay.

Chapter 5 Effect of FA on the molecular structure of PT

The structure of native PT and mutant PT samples before and after treatment with a range of FA concentrations, covering the concentrations employed in commercial pertussis vaccines, was analysed using SDS-PAGE, SEC, circular dichroism, analytical ultracentrifugation (AUC) and SEC multi-angle laser light scattering (SEC/MALLS). The effects of FA on protein conformation were explored using fluorescence spectroscopy.

Chapter 6 Effect of FA on PT epitopes and murine anti-PT immune response

The antigenic properties of FA-treated native and mutant PT were analysed by assessing the binding of a panel of mAbs to the samples by

ELISA and SDS-PAGE. FA-treated native PT and mutant PT samples were also used to immunise mice, and total IgG and IgG subclasses in the sera were quantified, as well as the serum neutralising capacity for PT using CHO-cells.

Chapter 7 Effect of FA on toxic biological activities of native PT

The toxic activity of the FA-treated native PT preparations was measured by four methods, namely ADP-ribosylation assay, CHO-cell neutralisation assay, leukocyte proliferation assay and histamine sensitisation assay.

Chapter 8 Discussion

A summary and discussion of the main results.

2.1**General Materials**

PBS: The phosphate buffered saline (PBS) used consisted of 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 and 8 mM Na_2HPO_4 , (AnalR reagents; BDH, Poole, Dorset, UK), pH 7.5, unless otherwise stated.

De-ionised water: All water used in experiments, for making solutions and buffers for samples, chromatography and dialysis, was de-ionised by ultra-violet (UV) radiation using an ELGA water purification system (ESF-ELGA, Buckinghamshire, UK), to a resistivity quality of 18.2 M Ω -cm.

Dialysis: SpectraPor7 dialysis tubing (regenerated cellulose, 1 mL/cm, molecular weight cut-off 3000 Da; Pierce and Warriner, Essex, UK), closed with SpectraPor dialysis tubing-clips, was used in all dialysis procedures.

Volume measurements: All volumes less than 5 mL were measured using Gilson pipettes, or multi-channel pipettes in 96-well plate assays where appropriate. Volumes greater than 5 mL were measured using plastic pipettes using an electric pipette filler. Large buffer volumes were measured in measuring cylinders. Percentage solute concentrations, including formaldehyde concentrations, were measured as weight by

volume (w/v) unless stated otherwise.

2.2 PT preparations

2.2.1 Commercial PT

Untreated PT and PT toxoids from five different manufacturers (A – E) were analysed using structural and immunological methods, in the study of the effect of different chemicals on PT (Chapter 4).

Untreated PT received from different manufacturers (A – E)

A. This PT sample was a genetically inactivated PT, which had a double amino acid substitution in the S1 subunit, Arg-9 → Lys and Glu-129 → Gly (PT-9K/129G) (Nencioni *et al.*, 1990), and is referred to herein as ‘mutant PT’. The sample was received from the manufacturer, at a protein concentration of 100 µg/ml in 50 mL volumes. It was sterilely aliquoted into 2 mL volumes in 2 mL plastic cryo-tubes, and stored at –20 °C, in 0.5 %, trehalose (crystalline (D+)trehalose dihydrate, Sigma), by adding 0.2 mL of a 50 %, trehalose stock solution to 1.8 mL PT solution, in PBS, and used within three years of receipt.

B. This PT sample was received from the manufacturer at a protein concentration of

191 µg/mL, in 5.8 mM NaH₂PO₄, 59.6 mM K₂HPO₄, 500 mM NaCl, at 4 °C, in 4 mL aliquots in 5 mL polypropylene screw-cap tubes. Analysis of the sample was within five years.

C. This PT sample was received from the manufacturer at a protein concentration of 200 µg/mL, in glycerol stabiliser (final concentration, 50 %, v/v), at –20 °C, in 2 mL aliquots in 2 mL plastic cryo-tubes. Analysis of this sample was within three years.

D. This PT sample was received from the manufacturer at a protein concentration of 200 µg/mL, in 50 mM Tris, 500 mM NaCl, 0.01 M glycine and glycerol stabiliser (final concentration, 50 %, v/v), at 4 °C, in 2 mL aliquots in 2 mL plastic cryo-tubes. Analysis of this sample was within three years.

E. This PT sample was received from the manufacturer at a protein concentration of 221 µg/mL, as an ammonium sulphate suspension, at –20 °C, in 2 mL aliquots in plastic cryo-tubes. Analysis of this sample was within three years.

Toxoids prepared by different chemical treatments

A. Low FA-treated mutant PT: The mutant PT was prepared for commercial use by treatment with a low concentration of FA (0.035 % (w/v); (Nencioni *et al.*, 1991)). It was received from the manufacturer in a 50 mL-volume, at a concentration of 200 µg/mL, and stored under the same conditions as the untreated mutant PT, but without

trehalose.

B. Glutaraldehyde and formaldehyde-treated PT: This sample was received from the manufacturer at a concentration of 483 µg/mL, in 150 mM NaCl, at 4 °C, in 4 mL aliquots in 5 mL polypropylene screw cap tubes. Analysis of this sample was within five years.

C. Glutaraldehyde-treated PT: This sample was received from the manufacturer at a protein concentration of 71 µg/mL and under the same conditions as the untreated PT.

D. Tetranitromethane-treated PT: This sample was received from the manufacturer at a protein concentration of 300 µg/mL and under the same conditions as the untreated PT.

E. Hydrogen peroxide-treated PT: This sample was received from the manufacturer at a protein concentration of 225 µg/mL, in glycerol (final concentration, 10 %, v/v) stabiliser, at –20 °C, in 2 mL aliquots in 2 mL plastic cryo-tubes. Analysis of this sample was within 3 years.

All untreated PT and toxoided PT preparations, from the different manufacturers (A – E) were dialysed (section 2.2.4) in PBS before any analysis and kept on ice during experiments. The different toxoids are referred to herein as toxoids A – E.

2.2.2. Native and mutant PT: For FA studies, and NAD⁺ binding studies

The effect of FA on native and mutant PT was studied using physico-chemical methods (described in Chapter 5), and immunological techniques (described in Chapter 6). Mutant PT and native PT were also used to study the NAD⁺ binding properties of PT using intrinsic fluorescence spectroscopy, (described in Chapter 3).

Native PT

This PT is the only sample referred to as ‘native PT’ throughout the study. Native PT was received from manufacturer D at a protein concentration of 200 µg/mL, with glycerol stabiliser (final concentration, 50 %, v/v), in sterile 1 mL aliquots, in 1 mL-glass ampoules. This sample was stored at 4 °C and used within 5 months, or was stored at –20 °C and used within 1 year.

Mutant PT

Untreated mutant PT was from manufacturer A, (described in section 2.2.1). Newly received sample was stored at 4 °C and used within 1 year of receipt; or stored at –20 °C (as described in section 2.2.1).

The mutant and native PT samples, stored at either 4 °C or at –20 °C, were dialysed (section 2.2.4) in PBS, and checked for consistency with previous analytical results by

SEC (section 2.3.4) and fluorescence spectroscopy (section 2.3.8) before each analysis or FA-treatment. All PT samples were kept on ice during experiments.

2.2.3 FA-treatment of native and mutant PT

Mutant and native PT were treated with a range of formaldehyde (FA) concentrations, up to 1.0 % (w/v), which covers the range of FA concentrations used in the treatment of commercial PT antigens (Sato, Kimura, and Fukumi, 1984; Nencioni *et al.*, 1991). Within this range two groups of PT preparations, low and high FA-treated, were produced. The low FA-treatment, 0 – 0.5 %, involved a short incubation time of 48 hours, as used in the stabilising of mutant PT. The high FA-treatment, 0.25 – 1.00 %, involved a longer seven-day incubation time, as employed by some manufacturers in native PT detoxification (Sato, Kimura, and Fukumi, 1984; Petre *et al.*, 1996).

Low FA-treated mutant PT samples were used in the majority of the physico-chemical studies (Chapter 5) and in the *in vitro* immunological studies, (Chapter 6). The high FA-treated native and mutant PT samples were compared using physico-chemical studies (Chapter 5). They were also used in the murine *in vivo* immunological studies, designed to examine the immune response to the PT preparations, (Chapter 6). Low FA-treated native PT could not be used in mice due to its high toxicity.

Lysine was present as a quencher in the reaction mixture during FA-treatment. The lysine

prevents excessive non-specific FA damage to the protein by producing the reactive lysine intermediate, hydroxymethyl lysine, which reacts with amino acid side chains (Petre *et al.*, 1996; Tome and Naulet, 1981; Tome, Kozlowski, and Mabon, 1985; Means.G.E. and Feeney.R.E., 1971a). In this study the concentrations of lysine were chosen in order that lysine was in molar excess of FA. This is the case for all of the preparations with the exception of the low FA-treated 0.5 % FA-treated sample.

Low FA-treated PT: The reaction mixture (2 mL final volume) consisted of 0.5 μ M PT ($A^{0.1\%}_{280\text{nm}} = 0.06$), 0.05 M lysine (400 μ L of 0.25 M stock; L-lysine, free base, Sigma, Poole, Dorset, UK), and 0, 0.035, 0.01, 0.05, 0.1 or 0.5 % FA (from formalin (37 %, w/v, formaldehyde) ACS reagent; Sigma) in PBS, pH 7.5 and were incubated at 37 °C for 48 hrs.

High FA-treated PT: The reaction mixture in PBS (2 mL final volume) consisted of 1 μ M PT ($A_{280\text{ nm}} = 0.12$), 0.35 M lysine (0.383 g dissolved in the reaction mixture) and 0.25, 0.5, 0.7 or 1.0 % (w/v) FA, and was incubated at 37 °C for 7 days.

Reaction mixtures were prepared in 2 mL plastic cryo-tubes by first adding lysine to the PT then the FA and PBS and were mixed once gently, before incubation at 37 °C.

Immediately after incubation, the FA-treatment samples were dialysed (section 2.2.4) extensively to remove the highly absorbing hydroxymethylated lysine and any other solution adducts.

2.2.4 Dialysis of PT samples

The azide preservative was washed off the dialysis tubing by immersing in deionised water for 5 minutes and then rinsing with deionised water. Samples were transferred from the storage vials to the dialysis tubing using a glass Pasteur pipette and the ends of the tubing turned over before clipping. To remove storage buffer from PT samples, before use in experiments, dialysis was performed in 3 changes of 2 L of PBS, pH 7.5 at 4 °C, the second change over night, with constant stirring. To remove the highly absorbing solution adducts formed during FA-treatment, more extensive dialysis was performed in at least 3 changes of 4 L of PBS over 2-3 days, with constant stirring.

2.2.5 Protein concentration technique

Concentration of PT by Centricon-3 centrifuge concentration tubes (Amicon, Gloucestershire, UK) or vacuum dialysis gave poor recovery of protein. Newly dialysed PT samples were successfully concentrated by covering the sealed, sample-filled dialysis tubing with a sucrose polymer, Ficoll (Type 400-DL, Sigma), and leaving it to draw out the liquid. The sample-filled dialysis tubing was placed on a clean tray, covered to a depth of 0.5 cm with Ficoll and left at 4 °C. Ficoll was replenished every hour until the required volume reduction was achieved. This technique reduced the sample volume by ~ 1 mL every hour and gave ~ 90 % recovery.

2.2.6 Determination of protein concentration

2.2.6.1 UV absorbance

This technique was used routinely to check the concentration of PT preparations before analysis. PT samples were dialysed (section 2.2.4) in PBS, centrifuged in 1.5 mL plastic o-ring tubes (Sarsdedt, Leicester, UK) for 10 minutes at 13 000 rpm in a Sanyo-Micro Centaur microcentrifuge, (Sanyo, Illinois, USA) and the supernatant carefully taken off, with a Pasteur pipette, for protein determination. Absorbance spectra of PT samples were recorded between 190 nm and 370 nm with the PBS dialysate as the blank, in matching 1cm path-length 1 mL quartz cuvettes (Hellma, Essex, UK), using a Cecil 6000 Series spectrophotometer. The maximal absorbance, which ranged between 271 nm and 280 nm for the different PT preparations, was recorded and used in equation (1) to calculate the protein concentration. According to the Beer-Lambert law the concentration, c (M), of a substance can be determined from the maximal absorbance, A , the path-length, d (cm), and the calculated molar absorption co-efficient, ϵ at maximal absorbance (Mach, Middaugh, and Lewis, 1992) which for PT is $126502 \text{ M}^{-1} \text{ cm}^{-1}$. Following FA-treatment, the highly absorbing lysine-FA intermediate (hydroxymethylated lysine λ_{abs} , 210 – 220 nm) interfered with the protein absorbance band. Therefore preparations were extensively dialysed (section 2.2.4) to remove as much of this adduct as possible for accurate protein determination.

$$c = \frac{A}{\epsilon d} \quad (1)$$

2.2.6.2 Bicinchoninic acid (BCA) assay

The BCA assay was carried out to check for consistency with the UV absorbance method. Protein concentrations were determined for eight different preparations: untreated mutant PT (section 2.2.2), four FA-treated mutant PT samples (*Low FA-treated*, section 2.2.3) and commercial PT samples B, C and D (section 2.2.1) with concentrations in the range 20 to 100 $\mu\text{g/mL}^{-1}$. Results agreed very well ($\pm 2.5 \mu\text{g/mL}^{-1}$) with absorbance-determined values.

The Micro BCA Protein Assay Reagent Kit (Pierce and Warriner, Essex, UK) was used. Full instructions for preparation of the standard protein, bovine serum albumin (BSA), and BCA working reagent are described in the kit instructions. In brief, dilutions (0 – 200 $\mu\text{g/mL}$) of the BSA standard protein solution were made in PBS, and 150 μL of each BSA dilution, PT sample or PBS (blank) were pipetted into wells of a 96-well microtitre plate (Falcon). The working reagent (150 μL) was added to each well and the plate was covered, mixed on a plate shaker for 30 seconds and incubated at 37 °C for 2 hours. The absorbance at 570 nm was recorded, and the protein values corrected by subtracting the average blank value. A standard curve was prepared by plotting the corrected absorbance reading for each BSA standard against its concentration ($\mu\text{g/mL}$), and used to determine the protein concentrations of the PT samples.

2.3 Structural techniques

2.3.1 Computer molecular modelling of the S1 subunit of PT

Three different models of the S1 subunit of PT were generated: non-modified, 'activated' and 'activated' with NAD⁺-bound.

2.3.1.1 Retrieving and visualising the S1 subunit model

The X-ray crystal structure of PT used in the molecular simulations, was obtained from the Research Collaboratory for Structural Bioinformatics, Protein Data Bank (RCSB, PDB) ((Berman *et al.*, 2000); <http://www.rcsb.org/pdb/>), PDB code: 1PRT (Stein *et al.*, 1994a). Visualisation and manipulation of the S1 subunit of PT was performed using the insightII programme (Molecular Simulations Inc, San Diego, California, see <http://www.accelrys.com>) on a Silicon Graphics Octane workstation (SGI, Mountain View, California, USA).

2.3.1.2 Molecular mechanics simulations

Energy minimisation calculations were carried out using the AMBER (Assisted Model Building with Energy Refinement) programme suite and forcefield (Cornell *et al.*, 1995). The effect of solvent was modelled using a generalised Born approach (Srinivasan *et al.*, 1998). For ‘activation’ and NAD⁺ docking, energy minimisation calculations were typically carried out using 500 steps of steepest descent before switching to a conjugate gradient method.

Activation

With the AMBER modelling programme, a neutral cysteine not involved in a disulphide bond is denoted by the residue name CYS. A cystine involved in a disulphide bond is denoted by the residue name CYX. Hence ‘activated’ PT, in which the Cys-41 – Cys-201 disulphide bond is broken, was modelled using the former residue name and non-‘activated’ PT using the latter.

Docking of NAD⁺

Partial atomic charges for the nicotinamide ring of NAD⁺ were computed using the Jaguar quantum chemistry programme (Schrodinger Inc, Portland, Oregon, USA), following structure optimisation using a HF/6-31G* basis set. Partial atomic charges for other parts of the NAD⁺ molecule, and for the protein, were taken from those previously reported for known residues in AMBER (Cornell *et al.*, 1995).

Docking of NAD⁺ to PT (with Cys41-Cys201 cleaved) was carried out on the basis that PT and diphtheria toxin (DT) share five homologous residues in their putative catalytic

sites (Stein *et al.*, 1994a), together with the existence of an X-ray crystal structure for the DT-NAD⁺ complex (PDB ID: 1TOX; Bell and Eisenberg, 1996). An initial attempt was made to position NAD⁺ in the active site of PT by superimposing DT-NAD⁺ on PT using C α atom coordinates of homologous amino acids. However, this led to part of a PT α -helix (amino acids 185 – 210) penetrating the ribose ring of the NAD⁺, which is a physically unrealistic artefact.

A minimisation scheme to dock NAD⁺ into the PT catalytic site was therefore adopted, which allowed flexibility in both the protein and NAD⁺ molecules. A locally developed programme was used to find all atom-atom distances of less than 15Å between the C α atoms of the five conserved residues in DT and non-hydrogen atoms of NAD⁺, in the crystal structure of the complex. These distances were used to obtain distance restraints between the NAD⁺ atoms and the C α atoms of the corresponding five conserved residues in PT. A total of 151 distance restraints were generated by this procedure. The restrained minimisation calculation was started from several different initial locations for the NAD⁺. The initial location giving the best agreement with the restraints was then used for any subsequent calculations.

Analysis of models

For all three S1 models, the dihedral bond angles, ϕ (defined by atoms N' – C – C α – N), ψ (defined by atoms C – C α – N – C') and χ_1 (defined by atoms N – C α – C β – C γ etc) were measured for each residue using insightII. In addition, the relative percentage solvent accessibility of each amino acid side chain (relative to X-Gly-X) was determined

using the NACCESS programme (Hubbard and Thornton, 1993). The degrees of rotation of each dihedral bond angle, and the change in amino acid accessibility were calculated after 'activation' and after NAD^+ binding to 'activated' PT.

2.3.2 Reduction of PT and detection of free sulphydryls

The number of free sulphydryls liberated with chemical reduction of mutant PT (section 2.2.2), was determined based on the method of A. Habeeb (Habeeb, 1972). Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid) or DTNB; Sigma) liberates 1 mole of 2-nitro-5-thiobenzoate ($\epsilon = 14150 \text{ M}^{-1}\text{cm}^{-1}$, 412 nm, in non-denaturing solution) from reaction with one mole of protein sulphydryl. The absorption, at 412 nm, of reduced PT in the presence of DTNB was measured to determine the number of disulphide bonds broken during reduction. Incubation of PT with a ten-times molar excess (260 μM) of dithiothrietol (DL-dithiothrietol (DTT), Sigma) at 4 °C overnight did not liberate any free sulphydryls, but the following more rigorous treatment with 4000 molar excess DTT was successful.

A reaction mixture (2 mL), with a final concentration of 90 $\mu\text{g/mL}$ PT (1.8 mL, 100 $\mu\text{g/mL}$ PT) and 100 mM DTT (200 μL , 1M stock) in PBS, pH 7.5, was incubated at room temperature for 2 hours in a 2 mL plastic cryo-tube. DTT was removed by dialysis in three changes, one overnight, of 4 L 0.1 M phosphate buffer (5.3 mL, 0.2 M NaH_2PO_4 + 94.7 mL 0.2 M Na_2HPO_4 in 200 mL deionised water), pH 8.0. To determine the number

of free sulphydryls formed as a result of disulphide bond reduction, DTNB at a final concentration of 260 μ M (from 2.6 mM stock solution) in 0.1 M phosphate buffer, pH 7.0 (39.0 mL, 0.2 M NaH_2PO_4 + 61.0 mL, 0.2 M Na_2HPO_4 in 200 mL deionised water) was added to 1 mL of the dialysed PT solution. Absorbance at 412 nm of the PT plus DTNB solution was measured after 15 minutes and checked again after 1 hour, against a blank solution containing 260 μ M DTNB in dialysate buffer in matching 1 mL, 1 cm path-length Hellma cuvettes. The concentration of 2-nitro-5-thiobenzoate, and thus the concentration of free sulphydryls, was determined.

2.3.3 SDS-PAGE

Commercially detoxified PT samples, B, C and E (section 2.2.1), and mutant PT untreated and treated with 0.035, 0.1 and 0.5 % FA (section 2.2.3), were separated by SDS-PAGE, prior to Coomassie blue staining (as described in this section) or to Western transfer for immunoblotting with Mabs (section 2.4.6.1). Identical untreated mutant PT samples (five per gel) were also separated for immunoblotting with anti-PT mouse sera, in order to probe subunit-specific antibodies (section 2.4.6.2). Gels, buffers, molecular weight markers and power pack were from Novex (Invitrogen Ltd, Paisley, Scotland, UK).

Procedures for setting up, running and removing the gel were carried out as described in the *Novex pre-cast gel instructions* booklet. For sample preparation, concentrated Tris-

glycine SDS sample buffer (five-times concentrate) and water were added to each PT sample ($\geq 200 \mu\text{g/mL}$) to give a final mixture ($40 \mu\text{L}$) containing $\sim 6.5 \mu\text{g}$ PT, 0.05 M tris-HCl, 10% (v/v) glycerol, 2% SDS and 0.05% bromophenol blue; plus 2% (v/v) β -mercaptoethanol for reducing gels. This mixture was heated in a 0.5 mL “O”-ring tube, at 80°C , for 10 minutes and $37 \mu\text{L}$ of the mixture ($5 - 6 \mu\text{g}$ PT) was applied to a pre-cast 14% tris-glycine acrylamide 10 well, 1.5 mm thick gel. The samples were subjected to electrophoresis in tris-glycine SDS running buffer (ten-times concentrate, diluted in deionised water to 1 L to give 0.29% w/v tris base, 1.4% w/v glycine and 0.1% w/v SDS), at a constant 125 V for about 90 minutes with an initial current of $30\text{-}40 \text{ mA}$, decreasing to $8\text{-}12 \text{ mA}$ at the end of the run.

For protein detection by Coomassie blue staining, the gel was immersed in stain (0.125% (v/v) Coomassie Blue R-250, 50% methanol and 10% acetic acid in deionised water) and shaken for one hour. The gel was shaken in destain I, (50% methanol, 10% acetic acid) for 1 hour then destain II (7% acetic acid, 5% methanol) until the required colour contrast was obtained. Images of the gels were taken using a UVP (Ultra Violet Products, Cambridge, UK) camera in a UVP white/UV transilluminator cabinet and visualised using Labworks analysis software for Windows.

Generally two molecular weight marker protein mixtures, Mark12TM (myosin, 200 kDa ; beta galactosidase, 116.3 kDa ; phosphorylase b, 97.4 kDa ; bovine serum albumin, 66.3 kDa ; glutamic dehydrogenase, 55.4 kDa ; lactate dehydrogenase, 36.5 kDa ; carbonic anhydrase, 31 kDa ; trypsin inhibitor, 21.5 kDa ; lysozyme, 14.4 kDa ; aprotinin, 6 kDa ;

insulin B-chain, 3.5 kDa; insulin A-chain, 2.5 kDa) or MultiMark TM (myosin; phosphorylase B; glutamic dehydrogenase; carbonic anhydrase; myoglobin, 30 kDa; lysozyme, aprotinin, insulin), were run on each gel. For the immunoblotting experiment, designed to probe murine sera for PT antibodies (section 2.4.6.2), five markers were run on each gel, arranged in alternation with five identical reference PT runs.

A standard curve was prepared, by plotting the molecular weights of the marker proteins against their relative mobility (distance of marker protein migration/distance of tracking dye migration), and used to estimate the relative protein molecular weights (M_r , kDa) of the PT protein bands.

2.3.4 Size exclusion chromatography with ultra-violet absorbance detection (SEC/UV)

Commercial toxoids, A – E (section 2.2.1), high and low FA-treated mutant and native PT samples (section 2.2.3) were analysed by FPLC and the molecular weights of eluting species were determined. This technique was also used to check mutant and native PT samples for consistency before each new experiment. In addition, SEC fractions were collected for fluorescence analysis.

The FPLC system (Pharmacia, Milton Keynes, UK) consisted of twin pumps, an injection valve (3-port solenoid) fitted with a 200 μ L loop and a Superdex 200 or Superdex 75 HR

10/30 gel filtration column, separation ranges 10 – 600 kDa and 3 – 70 kDa, respectively. PT samples were prepared for SEC by centrifugation in plastic “O”-ring tubes using a Micro Centaur microcentrifuge, at 13 000 rpm for 10 mins. Untreated PT and 0.035, 0.1 or 0.5 % FA-treated PT samples (containing 16.5 µg PT in a 220 µL volume) were loaded on to the column and eluted at ambient temperature in PBS, at a flow rate of 0.5 ml/min. Absorbance was monitored at 214 nm, using a zinc lamp, and 280 nm, using a mercury lamp, and the data were collected using FPLC Director software for OS/2.

The columns were calibrated using tyrosine (D,L-tyrosine, Sigma) plus a mixture of proteins from the Sigma molecular weight marker kit (carbonic anhydrase, 29 kDa; bovine serum albumin, 66 kDa; alcohol dehydrogenase, 150 kDa; beta-amylase, 200 kDa; apoferritin, 443 kDa; thyroglobulin, 669 kDa; and Blue Dextran, ~ 2000 kDa). Small quantities of each marker protein were mixed together in deionised water, dissolved, spun in a microcentrifuge, the supernatant aliquoted into 250 µL volumes in 0.5 mL eppendorf tubes, stored at –20 °C and used within 1 year. A calibration run was performed before each FPLC experiment, and the spectra compared with previous runs to check for variation. Only SEC data run on the same day, or which had superimposable calibration spectra, were collated and presented. A standard curve was prepared, by plotting the relative molecular weights of the standard proteins against their elution volumes, and used to determine the molecular weights (kDa) of the eluting PT protein species. The exclusion volume was 8 mL and the total volume was 21 mL for both the Superdex 200 and Superdex 75 columns.

2.3.4.1 SEC fractions

Fractions of FA-treated mutant PT (0.5 mL) were collected in 1.0 mL eppendorf tubes using a Pharmacia fraction collector linked up to the FPLC software. Fractions of the same peak were pooled and fluorescence measurements taken (section 2.3.8).

2.3.5 Size Exclusion Chromatography/ Multi-angle Laser Light Scattering (SEC/MALLS)

Mutant PT samples, treated with 0.035, 0.05 and 0.1 % FA (section 2.2.3), were analysed by SEC/MALLS and the molecular weights of eluting species were determined.

The HPLC system consisted of a Waters 590 Solvent Delivery Module (Waters, Watford, UK), a Rheodyne injection valve (Model 7125) fitted with a 100 μ L loop (Rheodyne, St. Louis, Mississippi) a guard column (GFC-Polysep P, 75x7.8mm; Phenomenex, Macclesfield, UK) and two analytical columns – TSK G3000PW + TSK G4000PW (Anachem, Luton, UK). Columns were eluted with PBS buffer, pH 7.5 (PBS tablets, Sigma). The eluent was pumped at a flow rate of 0.8 mL/min at ambient temperature. Samples were injected (100 μ L) at a concentration of 478, 505 and 525 μ g/mL for 0.035, 0.05 and 0.1 % FA-treated PT samples, respectively. Scattered light intensities of the eluted fractions were measured using a Dawn F multi-angle light scattering photometer (Wyatt Technology, Santa Barbara, CA) and concentrations were measured using an

Optilab 903 interferometric refractometer (Wyatt Technology, Santa Barbara, CA). The determination of molecular weights and molecular weight distributions by SEC/MALLS are described in (Wyatt, 1993) and were carried out using ASTRA software for Windows. Light scattering data from 12 detectors, at angles 44.6° – 158.2° , were used in the analyses. The dn/dc value (specific refractive index increment) for a commercial formaldehyde-treated tetanus toxoid was determined to be 0.22 mL/g and used for the pertussis toxin molecular weight calculations in this experiment.

2.3.6 Analytical ultracentrifugation (AUC)

Mutant PT samples, untreated and treated with 0.05 and 0.1 % FA (section 2.2.3), were analysed by sedimentation equilibrium AUC. Solute distributions were examined and weight average molecular weights determined.

Equilibrium solute distributions of PT samples were determined in a Beckman Optima XL-A analytical ultracentrifuge (Beckman Coulter, High Wycombe, UK) at 4°C , in a standard 1.2 cm path-length, 200 μL cell. Also in a 1.2 cm path length quartz cuvette, the concentrations of the samples were determined. Three different concentrations of each sample were analysed: untreated PT, 152, 221 and 291 $\mu\text{g/ml}$ ($A_{271\text{nm}}$ 0.22, 0.32 and 0.42); 0.05 % FA-treated PT, 118, 186 and 242 $\mu\text{g/ml}$ ($A_{271\text{nm}}$ 0.17, 0.27 and 0.35); and 0.1 % FA-treated PT, 69, 173 and 228 $\mu\text{g/ml}$ ($A_{271\text{nm}}$, 0.10, 0.25 and 0.33) in PBS dialysate, pH 7.4. Distributions were recorded with scanning

absorbance optics (in radial step sizes of 0.001 cm) at three rotor speeds, 15 000, 25 000 and 35 000 rpm. A true optical baseline was determined by sedimenting the protein from the bulk of the solution volume at 47 000 rpm.

The data were initially analysed using the data-fitting programme Origin, which uses models describing the sedimentation equilibrium process. Fits to the data with a 1 or 2 ideal species model were poor and therefore gave unreliable molecular weight estimates. The data were instead successfully analysed with the model-independent computer programme MSTAR (Cölfen and Harding, 1997) which calculates the M^* parameter. At the cell base, M^* is equal to the apparent weight average molecular weight ($M_{w, app}$).

2.3.7 Circular dichroism (CD)

CD spectra, of native and mutant PT (section 2.2.2), were obtained using a Jasco J-720 spectropolarimeter and analysed using the Jasco software for Windows (Jasco, Great Dunmow, UK). Native (750 $\mu\text{g/mL}$) and mutant PT (382 $\mu\text{g/mL}$) samples were dialysed in 3 changes of 2 L, PBS buffer or 42 mM K_2HPO_4 , 8 mM NaH_2PO_4 buffer, pH 7.6 (to obtain data from 180 – 195 nm, without the interference from chloride ions). They were analysed by near (250 – 320 nm) and far-UV (180 – 260 nm) CD in 1 cm and 0.02 cm path length cuvettes respectively. The sensitivity was set at 10 mega degrees for near UV and 50 mega degrees for far UV scans. The following settings were used: band width, 1 nm; response time, 1 second; scan speed, 50 nm/min; and step resolution, 0.5 nm.

Spectra were corrected for dialysate buffer blank and molarity and were expressed as molar circular-dichroic absorption ($\Delta\epsilon$). The spectropolarimeter instrument was calibrated according to the *Jasco Instruction Manual*.

2.3.8 Fluorescence spectroscopy

Commercially detoxified PT samples, A – E (section 2.2.1) and high and low FA-treated mutant and native PT samples (section 2.2.3) were analysed by fluorescence spectroscopy to analyse their protein secondary structure. This technique was used to check mutant and native PT samples for consistency with previous scans before each new experiment.

NAD⁺ binding to PT, before and after reduction with DTT (2.3.2), was also studied using fluorescence.

Intrinsic fluorescence of PT samples (10 - 50 $\mu\text{g/ml}$) was performed at 24 °C, using a Spex Fluoromax single photon-counting spectrofluorimeter (Jobin Yvon Ltd, Stanmore, UK). The temperature was kept constant at 24 °C with a water bath (model, Tempete TE-8D; Techne, Cambridge, UK), and the temperature inside the cuvette was measured before every fluorescence scan using a thermometer probe. The excitation wavelength was set at 280 nm or 295 nm and fluorescence emission was scanned in the range 260 – 500 nm, in 1 nm increments, with a one second integration time. The samples were analysed in a 1 cm path-length, 1 mL quartz cuvette (Hellma). The excitation band pass was set at 4.25 nm and the emission band pass at 4.50 nm. Data were recorded and

corrected using the Datamax software (Spex, Yobin Yvon Ltd) for Windows.

Emission spectra were corrected by subtracting the spectrum of the PBS baseline. The inner filter effect (absorbance of excitation light by the sample) was minimal at these low protein concentrations and was therefore not corrected for.

Water Raman excitation and emission spectra were recorded before each experiment and the fluorescence profile of the incident room light and/or of a fluorescent standard (NATA) were checked monthly according to the procedure detailed in the *Spex FluoroMax Spectrofluorimeter* instructions.

2.3.8.1 NAD⁺ binding titrations

The NAD⁺ binding affinities (K_d) of mutant PT, before and after reduction (section 2.3.2), and of native PT were determined by measuring fluorescence quenching with increasing additions of NAD⁺.

NAD⁺ (β-NAD, non-reduced; Catalogue Number, N6522; Sigma) was added to the PT sample (1mL, 50 µg/mL) in the cuvette using a 10 µL glass syringe (Microlitre syringe, Hamilton Company). Volumes of 1 – 5 µL of a 0.002 M or 0.02 M stock solution of NAD⁺ in PBS, were added to give final concentrations of 2 – 350 µM in 1 mL. After each addition of NAD⁺ the sample was mixed using a magnetic stirrer and fluorescence scans were performed exactly as described above.

The maximum tryptophan fluorescence intensity, in counts per second, was recorded for each scan. A titration using PBS was also carried out and the change in fluorescence intensity due to dilution of the sample or to photo-bleaching over the time course of the titration was found to be negligible. However, it was necessary to correct for the non-specific decrease in fluorescence intensity due to NAD^+ absorbing the exciting light (inner filter effect). In a separate titration, the fluorescence intensity of a tryptophan derivative n-acetyl tryptophanamide (NATA), which fluoresces but does not bind NAD^+ , was recorded on each addition of NAD^+ (as added to PT). Firstly, the fluorescence values for NATA were normalised so that the initial fluorescence intensity maximum of NATA was equal to that for PT (before any NAD^+ addition). Secondly, the PT fluorescence intensities (F_{PT}) at each NAD^+ concentration were corrected ($F_{\text{corr PT}}$) by multiplying them by the ratio of normalised initial fluorescence intensity for NATA with no NAD^+ ($F_0 \text{ NATA}$) over the normalised fluorescence intensity for NATA at the corresponding NAD^+ concentration ($F \text{ NATA}$). That is, $F_{\text{corr PT}} = F_{\text{PT}} * F_0 \text{ NATA} / F \text{ NATA}$.

The K_d values for NAD^+ binding were determined using the programme EqFit5 (kindly provided by Dr Stephen R. Martin, National Institute for Medical Research, Mill Hill, UK) which carries out a non-linear least squares fit of the corrected data to estimate the end point of the titration and thus the K_d . The programme also calculates the χ^2 values for each data fit. The % fluorescence quenching from the initial fluorescence, before addition of NAD^+ , to the final end point fluorescence was also calculated.

2.4 Immunological techniques

The effect of FA and chemical toxoiding on the antigenic properties of PT was investigated. The PT preparations were analysed, before and after chemical treatment, to assess the quality and quantity of their subunit epitopes using mAbs in immunoblotting and ELISA. All of the PT preparations were also used to immunise mice to assess the effects of the various chemical treatments on the immune response. The immunogenicity of the preparations in mice was assessed by assaying the total IgG, IgG1 and IgG2a response using ELISA. Immunoblotting was used to assess the presence of anti-subunit antibodies. In addition, the quality of antibody response was determined by assessing the ability of the sera to neutralise the toxic effects of an active reference PT on CHO-cells. The protective efficacy of the PT preparations was also assessed using an aerosol challenge assay.

2.4.1 Sterile conditions

Sterile assays and sample preparation were carried out in a Class II sterile cabinet using sterile plastics and reagents throughout, and 70 % ethanol solution in water was used regularly to disinfect surfaces.

Buffers and media required for sterile assays and mice immunisations were sterilised in 100 mL volumes in 150 mL glass bottles (unless otherwise stated) by autoclaving at a

minimum of 15 lbs at 121 °C for a minimum of 15 minutes, stored at 4 °C, and used within 1 ½ years.

PT and control samples used for CHO-cell assays and mice immunisations were sterile filtered, before dilution, through a low protein binding, 0.22 µm pore size, syringe filter (polyethersulfone, 13 mm membrane diameter; Nalgene).

2.4.2 PT preparations used

Commercial toxoids and FA-treated mutant and native PT preparations are analysed in the experiments described in the following sections (sections 2.4.3. – 2.4.8) and have been described previously (sections 2.2.1 and 2.2.2).

2.4.3 Reference PT and anti-serum

In immunoblotting assays, untreated mutant PT (section 2.2.2) was used as the reference; in ELISA, an active PT preparation (code P57, in-house reference from a commercial source) was used; and in the CHO-cell toxicity, PT neutralisation, histamine challenge and leukocyte proliferation assays, another active PT preparation (code 90/518, NIBSC reagent) was used.

In all anti-PT serum assays, the serum raised against PBS or PBS plus alum was the negative control, and the 1st International Reference Preparation (IRP) of mouse anti-PT serum (code 97/642) was used as the positive control.

2.4.4 Preparation of mAbs specific for PT subunits

Murine mAbs specific for epitopes on S1 – S5 were in the form of ascites fluid, or as supernatants produced from hybridoma cell lines (Sato *et al.*, 1984b).

2.4.4.1 mAbs in murine ascites

For immunoblotting experiments, mAbs 1B7 (S1), 9G8 (S2), 7E10 (S3), 9C6 (S4) and 7E3 (S5) were used in the form of concentrated murine ascites fluid, kindly donated by Dr Yuji Sato (National Institute of Health, Japan) (Sato and Sato, 1990; Sato, Sato, and Ohishi, 1991).

2.4.4.2 Production of mAbs from hybridoma cell culture

For ELISA, mAbs 1B7 (S1), 3A12 (S2), 7E10 (S3), 11E6 (S2/3), 7F2 (S4) and 7E3 (S5) were used as hybridoma supernatants produced from hybridoma cell lines. These antibodies are of the IgG isotype, and it is known that 1B7 is of the IgG2a subclass.

Preparation of chemical stock solutions for cell culture: All chemicals used in the growth of hybridoma cell lines were from Sigma unless stated otherwise. Basic medium, consisting of 2.25 g NaHCO₃, 10 mL sodium pyruvate (Gibco, supplied as 100 mM solution) and 0.2 mL gentamycin (supplied as 50 mg/mL solution), made up to 1L with IMDM (Iscoves modified Dulbecco's medium), was filter sterilised using a vacuum sterile filter unit (0.22 µm pore size, 500 mL Stericup; Millipore). Solutions of insulin (2 mL of 8mg/mL solution in 0.05 N HCl in deionised water) transferrin (2 mL of 1 mg/mL solution in PBS) and glutamine (10 mL of 3 % v/v solution in deionised water) were filter sterilised using a syringe filter (0.22 µm pore size, polyethersulfone, 13 mm membrane diameter; Nalgene). These stock solutions were all stored at 4 °C.

Complete medium: The final medium consisted of 157 mL basic medium, 30 mL foetal bovine serum, 2 mL glutamine solution, 0.2 mL 2-mercaptoethanol (Gibco, supplied as 50 mM), 0.2 mL insulin solution and 0.2 mL transferrin solution. The bulk complete medium was stored at 4 °C and appropriate amounts warmed in a water bath at 37 °C before use.

Culture of hybridoma cells: Cell culture was carried out under sterile conditions.

Hybridoma cell stocks were thawed in a water bath at 37 °C, added to 10 mL of complete medium in a centrifuge tube, spun at 80 g for 6 minutes and the supernatant carefully discarded. The cells were resuspended in 10 mL complete media, the suspension transferred into a 25 cm³ tissue culture flask and incubated at 37 °C in 5 % CO₂. When

cells were confluent (after 2 - 4 days), as checked under a light microscope with a magnification of 4- or 10-fold, 10 mL of cell suspension was transferred into a 75 cm³ tissue culture flask with 30 mL of complete medium. This was incubated again until confluent (after 2 – 4 days) and the cell suspension was split into two 75 cm³ flasks with 20 mL complete medium and incubated once more. Once these cells were confluent the suspension was transferred into 50 mL centrifuge tubes and spun at 80 g for 6 minutes. The supernatants were carefully removed, and those from the same hybridoma cell line were pooled, then aliquoted into 5 mL volumes and stored at – 20 °C. The mAb-binding was tested in each experiment by including a positive control on each ELISA plate.

2.4.5 Preparation of anti-PT mouse serum

Preparations of PT for immunising: Untreated mutant PT and FA-treated (0.25 – 1.00 %, *High FA-treated PT*, section 2.2.3) mutant and native PT, at an initial concentration of ~ 50 µg/mL, were prepared with and without alum adsorption (0.3 mL of a 2 % stock solution, Alhydrogel Al(OH)₃; Superfos, Denmark) in PBS under sterile conditions, to give 3 mL PT samples each of 10 µg/mL.

Before immunisation, all of the PT samples were analysed by the CHO-cell toxicity assay (section 2.5.2) and were found to contain < 300 ng active PT per 5 µg dose.

Immunisation: The mice (strain NIH, female, 5 – 6 weeks old; Harlem) were caged in

groups of five per sample and each mouse injected sub-cutaneously with PT sample (0.5 mL, containing 5 µg PT) or PBS (0.5 mL), with or without alum.

At 5 weeks post immunisation each mouse was terminally bled, by cardiac puncture, into a 1.5 mL eppendorf tube. The whole blood was left overnight at 4 °C for the erythrocytes to settle. The serum was then collected from the top of the tube and stored in a 0.5 mL eppendorf tube at –20 °C.

Two mice from the 0.25 % FA (no alum) sample group and one mouse from the 0.5 % FA (no alum) sample group died, 10 days after immunisation, therefore their sera was not available for analysis.

2.4.6 Immunoblotting

This technique was used to analyse the effect of different FA-treatments or commercial toxoiding on both PT subunit-specific epitopes using mAbs, and on antibodies specific for PT subunits in anti-PT murine serum.

Western transfer: Appropriate PT preparations had been subjected to SDS-PAGE by electrophoresis on Tris-glycine (14 %) acrylamide gels (described in section 2.3.3).

Blotting apparatus, power pack and NuPage transfer buffer (20 x concentrate, diluted with deionised water to 1 L to give 25 mM bicine, 25 mM bis-tris free base, 1.0 mM

EDTA, 0.05 mM chlorobutanol, pH7.2) were supplied by Novex.

Western transfer was carried out according to the *Novex NuPAGE electrophoresis system* booklet, and is described in brief here. After electrophoresis, the gel was removed from its cassette and placed carefully onto nitro-cellulose membrane (Hybond-C super; Amersham Life Sciences) wet with transfer buffer. The gel and membrane were sandwiched between sheets of transfer buffer-soaked filter paper, cut to gel-size, and placed into the blotting module packed tightly with transfer buffer-soaked sponges.

Protein transfer was carried out at a constant 25 V for 1 hour, with an initial current of ~150 mA decreasing to ~100 mA at the end of the run. Following transfer the membrane was rinsed with PBS four times then incubated, with 100 mL of 5 % Marvel solution in PBS, at 37 °C for 1 hour with slight shaking and then washed with PBS at least 5 times.

Immunoblot development: After the detection step, using mAbs or anti-PT serum (sections 2.4.6.1 and 2.4.6.2), each blot was washed thoroughly at least five times with ~ 15 mL PBS-Tween solution (0.05%, v/v, Tween 20 in PBS). Anti-mouse goat antibody peroxidase-conjugate (F_c specific, Sigma), diluted 1:1000 in PBS-Tween, was then added and shaken for ~ 2 hours. The membrane was washed thoroughly as before with PBS-Tween and developed using Sigma FAST DAB (3,3'- diaminobenzidine) tablets. Both tablets were dissolved in 15 mL deionised water, the membrane incubated in the solution at room temperature, with shaking, until the band colour developed (about 2 minutes) and washed with deionised water to stop the reaction. Care was taken not to over-develop the blot as this can cause a high background colour. Images of the blots were prepared as for

the SDS-PAGE gels (section 2.3.3).

2.4.6.1 Detection of PT subunit-specific epitopes using mAbs

To investigate the effect of different chemical treatments on PT subunit epitopes, blots of FA-treated PT preparations and toxoids were screened with mAbs 1B7 (S1), 9G8 (S2), 7E10 (S3), 9C6 (S4) and 7E3 (S5) (section 2.4.4.1).

Each blot, containing untreated mutant PT, 0.035, 0.1 and 0.5 % FA-treated mutant PT or commercial toxoids, B, C and E, and untreated native PT, was placed in a petri dish, fully coated with 2 mL of one of the mAb concentrated ascites fluids (section 2.4.4.1), covered and incubated at room temperature overnight. The blots were developed as described above.

2.4.6.2 Detection of antibodies specific for PT subunits in anti-PT murine serum

To investigate the effect of FA on the anti-PT subunit specific antibodies produced, Western blots of reference PT (untreated mutant PT, section 2.4.3) were screened with murine serum, raised against different FA-treated PT preparations.

In this experiment, the nitrocellulose Western blots, of five alternating reference PT and molecular weight markers, were cut into five strips. Each strip contained one reference PT and one marker run. They were each placed in separate petri dishes and fully coated with 2 mL of one of the serum samples, raised against PBS or FA-treated (0.25 – 1.00 % FA) mutant or native PT; or with the reference anti-PT serum preparation (all 1:3 in PBS). The petri dishes were covered and incubated at room temperature overnight, with gentle shaking. The blots were developed as described above.

2.4.7 ELISA

This technique was used in two different experiments. **A.** The subunit-specific epitopes of PT in FA-treated PT preparations were quantitatively assessed by screening the preparations using subunit-specific mAbs. **B.** The PT-specific IgG antibody in murine serum raised against the FA-treated PT preparations or the commercial PT toxoids was quantitatively assessed by identifying serum-binding to reference PT.

2.4.7.1 Preparation of ELISA plates

Coating and blocking plates: **A.** To investigate the effect of different chemical treatments on the quantity of PT subunit epitopes, 96-well plates were coated with untreated or FA-treated (0.10 % FA) mutant or native PT, or reference PT (P57; section

2.4.3). **B.** To investigate the effect of chemical toxoiding or FA-treatment on the quantity of PT-specific IgG antibodies in murine serum, the plates were coated with reference PT (P57; section 2.4.3).

The PT preparations for coating were diluted to 2 µg/mL with sodium carbonate coating buffer (from a 1 L stock containing 15 mM Na₂CO₃, 350 mM NaHCO₃, 0.05 % w/v azide), pH 9.7. The coating solution (100 µL) was pipetted into the appropriate wells of 96-well plates Plates (Nunc-Immuno plate, MaxiSorp surface; Nunc), the plates covered, incubated at room temperature overnight and then washed (described below). To block the plates, 100 µL of FCS diluent (10 % v/v foetal bovine serum, Life Technologies, in PBS-tween) was added to each well, incubated at room temperature, with gentle shaking, for 30 – 60 minutes and then washed.

Plate washing: The solution filling the plate wells was shaken out, the plate immersed in a box of PBS-tween to fill all wells and the PBS-tween shaken out. This process was repeated four times and the plate was dried off with paper towels ready for the next solution addition.

2.4.7.2 Detection of subunit-specific epitopes in PT preparations by mAbs

To investigate the effect of different chemical treatments on the quantity of PT subunit epitopes, plates coated with the different PT preparations (see A. section 2.4.7.1) were screened with PT subunit-specific mAbs 1B7 (S1), 3A12 (S2), 7E10 (S3), 11E6 (S2/3), 7F2 (S4) and 7E3 (S5) (section 2.4.4.2).

Primary antibody: The PT preparations were screened with mAbs, specific for epitopes on PT subunits (section 2.4.4.2), produced from hybridoma cell lines. Mab solutions, 1B7, 3A12 or 11E6 at a dilution of 1:1000 or 7E10, 7F2 or 7E3 at 1:100, were pipetted, in duplicate, into the top well of a column, and each solution diluted 1:2 down the plate in FCS diluent to give 100 μ L in each well. The last well of each row was left containing diluent only in order to determine background variation between wells. The plate was incubated at room temperature, with gentle shaking, for two hours and then washed.

Antibody-enzyme conjugate: Anti-mouse goat antibody peroxidase-conjugate (F_c specific, Sigma), 100 μ L of 1:1000 in PBS-Tween, was pipetted into each well, the plate incubated at room temperature, with gentle shaking, for two hours and then washed.

Substrate reaction: The peroxidase substrate consisted of 1.25 mL of 1% 3,3', 5,5'-tetramethyl-benzidine (TMB) in dimethylsulphoxide (DMSO; 0.015 g TMB in

1.5 mL DMSO; both Sigma) and 37 μ L hydrogen peroxide (supplied as 30 %, w/w solution; Sigma), slowly mixed with 125 mL acetate buffer in deionised water (from a 1 L, ten-times concentrate stock, 0.54 M $\text{NaCH}_3\text{COOH} \cdot 3\text{H}_2\text{O}$, 0.16 % v/v glacial acetic acid; both BDH). This substrate solution was prepared immediately before use, as it is light sensitive. Substrate solution (100 μ L) was pipetted into each well and incubated at room temperature, with gentle shaking, for 10 minutes. Dilute H_2SO_4 (50 μ L; 1 M, in deionised water) was then added to each well, to stop the reaction, and the absorbance, at 450 nm, of the solutions was read using a Multiskan MS plate reader and accompanying Genesis software for Windows (Labsystems). The data were subjected to statistical analyses (section 2.4.7.3).

2.4.7.3 PT-specific immunoglobulins (Ig), total IgG, IgG1 and IgG2a, in anti-PT mouse sera

To investigate the effect of FA on the quantity of PT-specific total IgG, IgG1 and IgG2a, in murine serum (see section 2.4.5) raised against different FA-treated PT preparations, plates coated with reference PT (see section 2.4.7.1 B) were screened with one of the test anti-PT serum or the reference anti-PT serum (section 2.4.3) and then anti-mouse IgG.

Primary antibody: Reference PT was screened with anti-PT sera raised against untreated mutant PT, FA-treated (0.25, 0.5, 0.7 or 1.0 %) mutant and native PT and PBS (negative control) (all with and without alum). Serum from each mouse per sample group

was individually assayed. Anti-PT reference serum was assayed in duplicate on each plate. Anti-PT serum solutions were pipetted into the top well in FCS diluent at a dilution of 1:1000, for total IgG and IgG1 determination, or 1:5, for IgG2a determination, and diluted in FCS diluent 1:3 down the plate to give 100 μ L in each well. The last well in each column was left containing diluent only to determine background variation between wells. The plates were incubated at room temperature, with gentle shaking, for two hours and then washed.

Antibody-enzyme conjugate: Total IgG was detected using goat anti-mouse total IgG peroxidase-conjugate (1:1000; Sigma) (as in section 2.4.7.1). IgG subclasses were detected using rat (strain LOU) anti-mouse IgG1 or IgG2a (isotype, IgG1, κ) biotin-conjugates (1:1000 or 1:200 respectively; Pharmingen). Each well was filled with 100 μ L of antibody conjugate at the appropriate concentration in FCS diluent. The plate was incubated at room temperature, with gentle shaking, for 1 ½ hours and then washed.

Substrate reaction: For detection of total IgG, peroxidase substrate was used (as in section 2.4.7.1). For detection of the IgG subclasses, streptavidin-horseradish peroxidase conjugate (100 μ L, 1:1000 in diluent; Pharmingen), was added to each well, the plate incubated at room temperature, with gentle shaking, for 1 hour and then washed. The peroxidase substrate (Sigma Fast o-phenylenediamine dihydrochloride tablets, in deionised water), 100 μ L, was pipetted into each well and the plate incubated in the dark, at room temperature, for 20 minutes. HCl (50 μ L; 3M, in deionised water) was then added to each well to stop the reaction, and the absorbance, at 492 nm, of the solutions

was read using a Multiskan plate reader. The data were subjected to statistical analyses (section 2.4.7.3).

2.4.7.4 Statistical analysis of ELISA data

ELISA data was obtained from the Multiskan plate reader in the form of an absorbance titration curve, as absorbance (at 492 nm) against dilution. The linear portion of each titration curve, for the test samples and the reference preparation, was analysed by parallel line assay, using an in-house statistical analysis programme (PLATE_RANDOM, NIBSC), which determines the sample titre relative to the reference, taking into account well-to-well variation. The negative controls were always found to give a titre of zero. No serum results were excluded from the statistical analyses. However, two serum samples from mice immunised with 0.25 % FA-treated PT (no alum) and one from the mice immunised with 0.5 % FA-treated PT (no alum) were not available for analysis, because these mice died ten days after immunisation.

The geometric mean, GM , and geometric standard deviation, GSD , (equations 2 – 4) were determined for duplicate mAb-binding titres (section 2.4.7.1) and for each group of five serum IgG titres (section 2.4.7.2). The GM was calculated for each group of results, $x_1 - x_N$, where N is the total number of values in the group (equation 2). From the geometric variance, GV , the GSD associated with each GM was determined (equations 3 and 4). The difference between two groups of data was accepted as statistically significant if $P \leq 0.1$, as determined with Students t-Test.

$$\log GM = \frac{\sum_{i=1}^N (\log x_i)}{N} \quad (2)$$

$$\log GV = \frac{\sum_{i=1}^N (\log x_i - \log GM)^2}{N} \quad (3)$$

$$GSD = \sqrt{GV} \quad (4)$$

2.4.8 PT-neutralising activity of anti-PT sera, using CHO-cells

Biologically active PT, with both a functional binding B-oligomer and catalytic S1 subunit (Pizza *et al.*, 1989; Loosmore *et al.*, 1993) affects the morphology of CHO-cells growing in a monolayer causing a characteristic clustering effect (Gillenius *et al.*, 1985). The ability for anti-PT sera to neutralise the toxic effects of PT on CHO-cells was assayed.

PT-neutralising activity of anti-PT sera was determined by pre-incubating a series of dilutions of the anti-PT sera with active reference PT (code 90/518; section 2.4.3) in a 96-well plate, adding the CHO-cells and observing the dilution of sera which caused inhibition of the clumping effect. Three serum samples from each group of five raised against 0.25, 0.5 and 1.0 % FA-treated PT (without alum) were randomly chosen for analysis. In addition, the positive control reference serum (see section 2.4.3) and negative control sera raised against PBS (without alum) were also analysed.

2.4.8.1 Preparation of CHO-cell suspension

CHO-cell culture media: Complete RPMI medium was used for CHO-cell culture and consisted of 90 mL incomplete RPMI 1640, 10 mL foetal bovine serum (Life Technologies) and 1 mL antibiotic antimycotic solution (from a 100 x solution containing 10000 Units penicillin, 10 mg/mL streptomycin and 25 µg/mL amphotericin; Sigma).

Preparing a confluent monolayer: CHO-cell culture was carried out under sterile conditions. CHO-cell (strain K1; ICN, Basingstoke, UK) stocks were stored in foetal bovine serum with 10 % (v/v) DMSO, in 2 mL cryo-tubes, in liquid nitrogen. A stock vial was removed from the liquid nitrogen, left at room temperature until only just thawed. The CHO-cells were immediately transferred into 20 mL complete RPMI medium in a 50 mL centrifuge tube and spun at 100 g, at 2 – 8 °C for 6 minutes. The supernatant was discarded and the cells resuspended in 50 mL complete RPMI medium, transferred to a 75 cm³ culture flask (Falcon) and incubated (2 – 4 days) at 37 °C, in 5 % CO₂ until confluent as checked under a light microscope using a 4- or 10-fold magnification.

Releasing and diluting cells: Following incubation, the medium was discarded and the cell layer carefully rinsed twice with PBS before releasing the cells from the flask surface by trypsination. Trypsin (5 mL, 0.25 %; Life Technologies) was added to cover the layer

of cells, the flask left at room temperature for 3 minutes, trypsin poured off and the flask incubated again at 37 °C for 5 minutes. Five mL of complete RPMI medium was added to the flask and the cells gently washed off the base of the flask. Cells in the suspension were counted using a haemocytometer and diluted with complete medium to a concentration of 2×10^4 cells/mL. This cell suspension was prepared in the same way for both the CHO-cell toxicity assay (section 2.5.2) and the CHO-cell neutralisation assay.

2.4.8.2 Preparation of neutralisation assay plates

The preparation of neutralisation assay plates was carried out under sterile conditions. RPMI media (25 µL) was pipetted into all wells of a 96-well plate. Each serum sample (25 µL, 1:10 in PBS), including negative (serum raised against PBS) and positive (97/642, 1st IRP) controls, was pipetted into the first well of a row and diluted 1:2 across the row. Active reference PT 90/518 (25 µL of a 16 ng/mL solution) was then added to each well, excluding one row. One row per plate was reserved to check the CHO-cell toxicity (section 2.5.2) of the active reference PT. In this row 25 µL, 64 ng/mL of active reference PT was pipetted into the first well and diluted 1:2 across the row, and another 25 µL of RPMI added to each well in the row instead of serum. The plate was gently shaken for a few minutes and then incubated at 37 °C for 3 hours. The CHO-cell suspension (200 µL) was then added to each well, the plate gently shaken for a few minutes and then incubated at 37 °C in 5 % CO₂ until there was sufficient level of cell growth to observe the morphological changes. This usually took 2 days and care was

taken not to over-culture the cells as they can recover from the toxin-induced changes (Gillenius *et al.*, 1985).

2.4.8.3 Interpretation of neutralisation assay plates

The plate was removed from the incubator and viewed on a light microscope at 4- or 10- fold magnification. The wells were examined across the plate for the presence of clustering as compared to the positive and negative controls. The titration end-point was taken as the highest serum dilution causing complete neutralisation of the toxin effect, resulting in unaffected cells (Gillenius *et al.*, 1985). The reciprocal of the titration end-point dilution factor for each serum sample was taken as the neutralising titre. The neutralising titres for the test sera were corrected for variation between plates relative to the reference antiserum titration on each plate. However, the plates were consistent and varied occasionally only by one dilution factor. The geometric mean and geometric standard deviation of the neutralising titre for each group of three sera were determined.

2.4.9 Aerosol challenge

Procedure

Groups of ten mice were immunised with one of the commercial toxoids (A – E), and subjected to aerosol challenge 30 days later using a live suspension of *B. pertussis* cells,

which were cultured on charcoal agar plates. The lungs from the mice were homogenised in lung tubes, plated out on charcoal agar plates and the number of viable cells per lung calculated.

Mice immunisation

Groups of ten mice (NIH) were immunised by the intraperitoneal route with 5 µg of one of the toxoids, A – E (described in section 2.2.1), or the positive control reference vaccine (in house reference, co-purified pertussis vaccine, code JNIH-3), in 0.5 mL saline (0.9 % NaCl, anhydrous), or with 0.5 mL saline alone, as the negative control.

Charcoal agar plates

A 5.1 % charcoal agar base (Oxoid, Hampshire, UK) solution (12.75 g in 250 mL water) was sterilised and allowed to cool to 50 °C. To this solution, 25 mL sterile defibrinated horse blood (Oxoid) was aseptically added, mixed and 15 – 20 mL aliquots were poured into 9 cm Petri dishes and stored at 4 °C for up to six weeks.

Preparation of *B. pertussis* suspension

B. pertussis cells (strain Wellcome 28) were stored in 200 µL aliquots, in a 5 % glycerol solution (v/v) in 1% casein solution, at -65 °C. Five days before aerosol challenge (day one), one aliquot of *B. pertussis* cells was taken from storage, allowed to thaw at room temperature and aseptically dropped onto two charcoal agar plates using a sterile glass Pasteur pipette. The plates were incubated for two days at 37 °C. On day three a visible amount of culture from these two plates were evenly streaked onto six fresh plates and

these were incubated at 37 °C for 18 – 24 hours. On day four, all of the culture was scraped from the six plates, resuspended in 3 mL of 1 % casein solution and this suspension dropped and spread onto six fresh plates (0.5 mL per plate), and these plates were incubated at 37 °C for 18 hours. On day five, the day of the aerosol challenge, all of the bacterial culture was resuspended in 6mL 1 % casein solution, 0.3 mL of this added to 100 mL 1 % casein solution and the suspension was adjusted to an optical density reading of 0.2 (absorbance at 625 nm), 40 000 bacteria/mL. This suspension was split between two 50 mL centrifuge tubes and kept on ice until required. This amount of suspension is sufficient to challenge 70 mice.

Lung tubes

Plastic O-ring conical tubes (Sarsdedt) were filled one third full with glass beads (BDH) and 1 mL of a 1 % casein solution (6 g NaCl (anhydrous) and 10 g casamino acid; BDH in 1 L of distilled water). The filled and capped tubes were sterilised and stored at 4 °C.

Aerosol challenge apparatus and procedure

The aerosol challenge apparatus and procedure were previously described by D. Xing and colleagues (Xing *et al.*, 1999), and are described briefly here. The day before challenge, 8 mL of 70 % ethanol solution was run through the aerosol system. This was repeated on the day of challenge, followed by 2 x 8 mL sterile water. A sterile nebuliser was then attached to the system for the start of the challenge. Each group of ten mice was placed in the metal restraint boxes, the nebuliser filled with 8 mL of challenge suspension and the airflow started. The mice were exposed to the aerosol challenge for five minutes.

Lung sampling

Five saline control mice were sampled two hours after challenge to check that the aerosol challenge was successful. All other groups were sampled seven days after challenge.

Lungs were removed following terminal anaesthesia intraperitoneal injection, and each lung placed in a lung tube. These were kept on ice until required. Each lung was homogenised in the lung tube using a mini beadbeater at a speed of 4600 rpm for ten seconds. The homogenate was placed on ice.

Determination of viable counts

A series of dilutions of each lung homogenate (neat, 10-, 100- and 1000-fold), were prepared in quadruplicate in a microtitre plate. Each was dropped (25 μ L) onto a charcoal agar plate so there were 16 spots per plate (four dilutions, each in quadruplicate). At a countable dilution, the total number of colony forming units (CFU) in each of the quadruplicate drops were added up to give the number of CFU per 100 μ L at that dilution. This count was then multiplied by the appropriate dilution factor and by 10 to give the total CFU count per lung (1 mL).

The geometric mean CFU count and geometric standard deviation (equations 2 – 4, section 2.4.7.4) for each group of ten mice was determined.

2.5 Determination of PT toxic biological activities

The toxic biological activities of 0.25 – 1.00 % FA-treated native PT preparations (*High FA-treated PT*, section 2.2.3) were determined using four different methods: CHO (Chinese hamster ovary)-cell toxicity assay, ADP-ribosylation assay and two murine *in vivo* tests, leukocyte proliferation and histamine sensitisation. These activities are all dependent on the functional B-oligomer (Loosmore *et al.*, 1993) and ultimately the functional catalytic activity of the S1 subunit (Nencioni *et al.*, 1990).

These FA-treated native PT samples were also incubated and examined for an increase in their toxic biological activities (toxicity reversion) by the CHO-cell toxicity assay and the ADP-ribosylation assay.

2.5.1 Reference PT

Reference PT used for the positive control in all four assays was an active PT preparation (code 90/518, NIBSC reagent). The negative controls used were PBS and mutant PT, which is devoid of catalytic activity and thus does not possess the toxic biological activities of CHO-cell toxicity, ADP-ribosyl transferase and leukocyte proliferation and histamine sensitisation (Nencioni *et al.*, 1990).

2.5.2 CHO-cell toxicity assay

Native PT causes a characteristic clustering effect of CHO-cells growing in a monolayer, (described in section 2.4.8). The concentration of biologically active PT in a FA-treated native PT preparation was determined by the CHO-cell toxicity assay by incubating CHO-cells with a series of concentrations of the sample, in a 96-well plate, and observing the end-point of the CHO-cell clumping effect.

CHO-cells were grown to confluence and a cell suspension prepared to a concentration of 2×10^4 cells/mL as described in the PT neutralisation assay (section 2.4.8).

2.5.2.1 Preparation of CHO-cell toxicity assay plates

Assay preparation was carried out under sterile conditions. Incomplete RPMI medium (25 μ L) was pipetted into all wells of a sterile 96-well plate (Microtest, flat bottom, tissue culture plate; Falcon). The active reference PT or FA-treated native PT preparations, neat and diluted 1:10 (in sterile PBS), were pipetted (25 μ L) into wells of the first column.

Samples and control were tested in duplicate. These solutions were diluted 1:2 across the plate using a multi-channel pipette. Two wells of every plate were left containing medium only, for the negative control. The CHO cell suspension (200 μ L) was added to each well, the plate gently shaken for a few minutes, and then incubated at 37 °C in 5 % CO₂ until there was sufficient level of cell growth to observe the morphological changes.

This usually takes 2 days and care was taken not to over-culture the cells as they can recover from the toxin-induced changes (Gillenius *et al.*, 1985).

2.5.2.2 Interpretation of CHO-cell toxicity assay plates

The plate was removed from the incubator and viewed on a light microscope at 4- or 10- fold magnification. The wells were examined across the plate for the presence of clustering as compared to the negative and positive controls. The titration end-point was taken as the highest dilution to cause complete clustering (Gillenius *et al.*, 1985). The minimum concentration ($\mu\text{g/mL}$) of active reference PT to cause clustering (cytopathogenic concentration) was calculated by multiplying its neat concentration added to the first column, by its titration end-point dilution. The concentration of biologically active PT ($\mu\text{g/mL}$) in the neat PT sample added to the first column, was determined by multiplying its end-point dilution factor reciprocal by the minimum cytopathogenic concentration. Average values for duplicate analyses are quoted as Tg active PT per 100 μg of total protein.

2.5.3 ADP-ribosylation assay

The ADP-ribosyl transferase activity of the FA-treated native PT preparations was determined by measuring the ribosylation of a fluorescent-tagged peptide substrate using

an enzymatic-HPLC coupled assay, based on the method of Dr Larry W. Whitehouse and co-workers (Cyr *et al.*, 2001). The PT preparations were activated, prior to the enzymatic reaction, and then added to the fluorescent-tagged peptide substrate in the presence of ribosylating reagent containing NAD^+ and ATP. The quantity of ADP-ribosylated fluorescent-tagged peptide substrate produced by each FA-treated native PT preparation was detected by HPLC, and the amount of active PT in each sample was calculated relative to the active reference PT.

The following reagents were of analytical quality and obtained from Sigma, unless otherwise stated.

PT activation buffer: Dithiothreitol (Aldrich) 200 mM in 0.5 M Tris HCl, pH 7.6.

Ribosylating reagent: The ribosylating reagent contained equal volumes of i, ii, and iii:
(i) 20 mM phenylmethylsulfonyl fluoride (PMSF, a serine protease inhibitor) containing 1.5 mg/mL lysophosphatidylcholine (LPC) in a 2:3 (v/v) solution of isopropanol and 0.1 M Tris HCl, pH7.6. This solution was prepared using stock solutions of PMSF (100 mM, dissolved in isopropanol) and LPC (10 mg/mL LPC, in a 9:1, (v/v) chloroform/methanol mix). When preparing the working PMSF/LPC solution, the organic solvent was removed from the LPC with a stream of nitrogen, prior to addition of the PMSF solution and Tris HCl buffer. The working PMSF/LPC solution was sonicated for one minute in an ultrasonic water bath before use.

(ii) 18 mM ATP in 0.1 M Tris HCl, pH 7.6

(iii) 3 mM NAD⁺ (β -NAD, as used in section in 0.1 M Tris HCl, pH 7.6

The ribosylating reagent was sonicated for one minute in a water bath before addition to the assay reaction mixture.

Enzyme substrate peptide: The enzyme substrate, a fluorescein caproic acid- (FAC) tagged G_{ai3} C20 peptide (FAC-*VFDAVTDVVIKNNLKECGLY*-COOH), was a gift from Dr Larry W. Whitehouse (Cyr *et al.*, 2001), and is referred to herein as the C20 peptide.

Enzyme Assay

A series of concentrations of active reference PT (0 – 0.4 μ g/mL) were prepared in duplicate in 2 mg/mL ovalbumin solution, for preparation of a standard curve. Dilutions of each FA-treated PT preparation (from two to five-fold dilutions of the neat 50 μ g/mL protein preparations), were made which kept the reaction within the linear range of the standard curve. The test PT preparations or references (20 μ L), were incubated under nitrogen with 5 μ L of PT activation buffer, for 10 minutes at 37 °C. To this mixture, ribosylating reagent (10 μ L) was then added, vortexed, nitrogen added, and incubated at 4 °C for 30 minutes. Then the C20 peptide substrate (5 μ L) was added, the mixture vortexed, nitrogen added and further incubated at 21 °C for six hours. The reaction was stopped by adding 40 μ L of a 1:1 (v/v) solution of 0.5 M ammonium acetate buffer, pH8.5, and isopropanol. The reaction mixture was stored at -20 °C until HPLC analysis was performed.

Reverse-phase HPLC

HPLC analyses were performed on a titanium and PEEK-lined Gilson binary pump system (Anachem), using a reverse-phase column (Shodex Asahipak, ODP50-4E (5 μ , 4.6 x 250 mm); Phenomenex). Detection was by a Gilson fluorescence detector, model 122 (Anachem). The HPLC system was operated, and data collected by the Gilson Unipoint software (Anachem).

The HPLC mobile phase was aqueous acetonitrile buffered with 10 mM ammonium nitrate, pH 8.5. Solvent A contained 80 % acetonitrile and solvent B contained 0 % acetonitrile. The elution gradient (based on % of solvent B) was 0-1 min, 75%; 1-2 min, 75-70%; 2-6 min, 70%; 6-8 min, 70-0%; 8-9 min, 0%; 9-11 min, 0-75 % and 11-20 min, 75% and the flow rate was 0.8 mL/min. Elution was monitored by detection of FAC-tag fluorescence emission at 515 nm (excitation wavelength (λ_{ex}) 480 nm). The non-modified C20 peptide eluted at 11.1 minutes and the ADP-ribosylated C20 peptide eluted at 7.8 minutes.

The area under the ADP-ribosylated C20 peptide peak was calculated for each concentration of active reference PT and was used to compose a standard curve for active PT. This was used to determine the quantity of active PT in each FA-treated native PT sample. The results were calculated as the average for each duplicate experiment and are expressed as μ g active PT/100 μ g protein.

2.5.4 Leukocyte proliferation test

Leukocyte proliferation activity of the FA-treated native PT samples, and active reference PT was studied by immunising mice with these PT preparations and counting the leukocytes in the resulting whole blood.

Immunising preparations: Four different FA-treated (0.25, 0.50, 0.70 and 1.00 % FA) native PT preparations and an untreated mutant PT preparation (negative control), each of 50 µg/mL, were diluted with PBSG (PBS with gelatin: 2g gelatin in 1 L PBS, sterilised) to give 3 mL PT preparations with a final concentration of 10 µg/mL. The active reference PT, of 1 µg/mL, was diluted with PBSG to give a final concentration of 200 ng/mL.

Mice immunisation: The mice (strain NIH, male, 5 – 6 weeks old; Harlan) were caged in groups of five per sample and were each injected intra-peritoneally, using a 5 mL syringe, with 0.5 mL of the FA-treated native or untreated mutant PT preparation (containing 5 µg), the active reference PT (containing 100 ng) or of PBSG only.

At three days post immunisation tail bleeds were taken from the mice. The mice were incubated at 35 °C for a few minutes prior to bleeding. The tail was pricked once with a sterile needle and approximately 100 µL of blood allowed to drip directly into an eppendorf tube containing 5 µL of 200 Units/mL heparin solution to give a final heparin concentration of ~ 10 Units/mL. The blood and heparin were mixed immediately and

leukocytes in the whole blood were counted, within 30 minutes of taking the blood, using a Coulter counter (Coulter Ac.T 8 Series Analyser; Beckman Coulter). The counts are expressed as the geometric mean for each group of five mouse serum samples, with geometric standard deviations (equations 2 – 4; section 2.4.7.4). The difference between two groups of data was accepted as statistically significant if $P \leq 0.1$, as determined with Students t-Test.

2.5.5 Histamine sensitisation test

Groups of five mice were immunised as for the leukocyte proliferation test (section 2.5.4).

Four days post immunisation the mice were challenged with histamine by intra-peritoneal injection of each mouse with 0.5 mL sterile histamine diphosphate solution (5.52 mg/mL; Sigma) in PBS. The mice were closely observed on injection, and the time of death, or culling of suffering mice, was recorded and regarded as positive for histamine sensitisation.

2.5.6 Toxicity reversion study

The toxicity reversion study was carried out on 0.5 and 1.0 % FA-treated native PT

preparations using CHO-cell toxicity and ADP-ribosylation assays.

A proportion of each FA-treated native PT sample (200 μ L of each at $\sim 50 \mu\text{g/mL}$) was removed from storage at 4 °C for the toxicity reversion study. These were incubated in 0.5 μ L, in polypropylene screw-cap tubes, at 37 °C for 11 days, and mixed manually twice every day. After incubation the preparations were stored at 4 °C overnight before performing the toxicity assays. The CHO-cell toxicity assay (section 2.5.2) and ADP-ribosylation assay (section 2.5.3) were then performed, on both the incubated samples and on the non-incubated samples (which had remained in storage at 4 °C), in duplicate. The quantity of active PT per 100 μg of protein was calculated, as determined in both techniques, for both the incubated and non-incubated PT preparations, as previously described (section 2.5.2 and 2.5.3).

Chapter 3

Effect of 'activation' and NAD⁺ binding on the structure of the S1 subunit of PT

3.1

Introduction

The S1 subunit of PT is an ADP-ribosyl transferase, which binds NAD⁺ and catalyses the transfer of the ADP-ribosyl moiety to the alpha subunit of host G-proteins. S1 contains 235 amino acids and has a molecular weight of 26 kDa with a secondary structure comprising 27 % α -helix and 24 % β -sheet (Appendix 1) (see Figure 1.4).

The known tertiary structures and sequences of ADP-ribosylating bacterial toxins, including PT, *Escherichia coli* heat-labile enterotoxins (LT1 and LT2), cholera toxin (CT), *Pseudomonas aeruginosa* exotoxin-A (PAETA) and diphtheria toxin (DT) (Stein *et al.*, 1994a; Sixma *et al.*, 1991; Sixma *et al.*, 1993; Allured *et al.*, 1986; Choe *et al.*, 1992) have been compared. In combination with site-directed mutagenesis these studies have highlighted a hydrophobic pocket predicted to bind NAD⁺ (Domenighini *et al.*, 1991; Domenighini *et al.*, 1994). In PT, several amino acids discovered to be important for assimilation of the NAD⁺ moiety in the catalytic action of S1, include Tyr-8, Arg-9, Phe-50, Thr-53, Glu-129 and Trp-26 (Pizza *et al.*, 1988; Locht, Capiou, and Feron, 1989; Cortina and Barbieri, 1989).

Of these, Trp-26 of the S1 subunit forms part of the predicted active site (Stein *et al.*, 1994a; Locht *et al.*, 1990) and is essential for maximum catalytic activity (Locht, Capiou, and Feron, 1989; Cortina and Barbieri, 1989). It is positioned on the bend of a hydrogen-bonded turn, flanked on either side by an extended α -helix (Appendix 1), the first section of which is conserved between PT, LT1, LT2 and CT (PT residues 8 – 25; Domenighini *et al.*, 1994). Analysis of the crystal structure (1PRT, Stein *et al.*, 1994a) shows that Trp-26 protrudes into the S1 molecule, and is only partially solvent exposed.

Trp residues are largely responsible for protein intrinsic fluorescence which is sensitive to the Trp environment, making intrinsic fluorescence spectroscopy a useful probe of PT conformation. Upon excitation at 280 nm, PT typically displays intrinsic fluorescence with Trp fluorescence maximum (F_{\max}) of ~ 337 nm, and a shoulder from tyrosine fluorescence at ~ 305 nm (Lobban, Irons, and van Heyningen, 1991). This fluorescence was shown to be specifically quenched upon NAD^+ binding, and the dissociation constant (K_d) of PT for NAD^+ determined by NAD^+ fluorescence quenching titrations (K_d μM) agrees well with both the K_d calculated by equilibrium dialysis, and the Michaelis constant for the NAD^+ -glycohydrolase activity of PT (Lobban, Irons, and van Heyningen, 1991). The proximity of Trp-26 in the putative NAD^+ binding site and its importance in the catalytic activity of PT suggests that it is Trp-26 fluorescence which is specifically quenched by NAD^+ , out of a total of six Trps in the holotoxin.

Also important for enzymatic activity is the cleavage of the putative active site disulphide bond Cys-41 – Cys-201, or 'activation', of the S1 subunit. This has been implied from

findings that reducing conditions are required for full catalytic activity *in vitro* (Moss *et al.*, 1986; Moss *et al.*, 1983). In addition, it has been speculated from observations of the crystal structure that cleavage of this bond would be a physical requirement for opening up the putative active site (Stein *et al.*, 1994a).

Two distinct binding sites for the G-protein substrate have also been localised in the S1 subunit, using recombinant S1 deletion peptides. One is within the amino terminal 180 amino acids (Finck-Barbancon and Barbieri, 1995) and the other is defined by residues 195 – 204 (Cortina and Barbieri, 1991). The residues 195 – 204 are part of an alpha-helix which is buried in the putative NAD-binding cleft (Stein *et al.*, 1994a; Krueger and Barbieri, 1994). A structurally homologous 'active site loop' has been purported to occlude the NAD⁺ binding site of LT, CT (amino acids 47 – 56) and DT (amino acids 39 – 46) (Pizza, Massignani, and Rappuoli, 2001). Moreover, in DT it is known to change conformation upon NAD⁺ binding (Bell and Eisenberg, 1996).

In the study of NAD⁺ binding to PT, predictions have been made from studying the crystal structure (Stein *et al.*, 1994a), which is based on non-activated PT. Two previous groups attempted to model NAD⁺ binding in the active site of PT. However, only the amino terminal amino acids ~ 2 – 189 of PT, and NAD⁺ fragments, were used in the simulations (Cummings, Hart, and Read, 1998; Scheuring, Berti, and Schramm, 1998). Despite the requirement for activation and NAD⁺ binding in the toxic ADP-ribosyl transferase activity of PT, there was no model previously available for 'activated' PT or the activated PT-NAD⁺ complex.

The aim of this study was to characterise the structural effects of 'activation' and NAD⁺ binding on the S1 subunit of PT, using fluorescence spectroscopy and computer modelling. Computer models for 'activated' PT and the NAD⁺-bound 'activated' PT complex are presented.

3.2 Results

3.2.1 Intrinsic fluorescence spectroscopy: Effect of 'activation' and NAD⁺ binding on mutant PT structure

Intrinsic fluorescence is a sensitive indicator of changes in protein conformation. Initially mutant and native PT structures, and their NAD binding properties, were compared by fluorescence. Mutant PT was then used in the remainder and majority of the study, for the investigation of changes in fluorescence upon 'activation' and NAD binding.

3.2.1.1 Comparison of native and mutant PT fluorescence

Native and mutant PT (~ 30 µg/mL) were subject to intrinsic fluorescence spectroscopy using a single photon-counting spectrofluorometer at an excitation wavelength (λ_{ex}) of

280 nm. The fluorescence was scanned from 260 to 500 nm and the base line fluorescence of the sample buffer (PBS, pH 7.5) was subtracted. To determine K_d values for the NAD^+ binding of mutant and native PT, their fluorescence spectra were recorded with the addition of increasing concentrations of NAD^+ (0 – 350 μM), and the fluorescence quenching titration data were corrected for the inner filter effect of NAD^+ and analysed using the EquFit5 programme (kindly provided by Dr Stephen R. Martin, National Institute for Medical Research, Mill Hill).

The fluorescence spectrum of mutant PT was found to be almost superimposable with that of native PT, both having an F_{max} from Trp fluorescence at 337 nm and a shoulder from Tyr fluorescence at ~ 305 nm (Figure 3.1). On NAD^+ binding, the Trp fluorescence was specifically quenched and the F_{max} remained unaltered, for both mutant (Figure 3.2A and B) and native PT (not shown). K_d values of 52 μM for native PT and 63 μM for mutant PT were obtained (Table 3.1) and are in close correlation with that obtained by Lobban and co-workers, of 37 μM for native PT (Lobban, Irons, and van Heyningen, 1991). These results demonstrate that mutant PT still binds NAD^+ , and with a similar affinity to native PT. On the basis of the similarity in protein structure and NAD^+ binding between native and mutant PT, mutant PT was chosen rather than native PT for safety reasons for the 'activation' and further NAD^+ binding fluorescence experiments.

Table 3.1 Kd for PT and NAD, by fluorescence spectroscopy

The dissociation constants (Kd) of different pertussis toxin (PT) preparations were determined by measuring the decrease in PT fluorescence spectroscopy with NAD concentration, at 24 °C. The data were corrected for the inner filter effect of NAD and analysed using the EquFit5 programme (kindly provided by Dr Stephen R. Martin, National Institute for Medical Research, Mill Hill) to give the Kd values, % fluorescence decrease and errors of the data fit (χ^2). *Lobban and co-workers carried out the binding experiment at 23 °C, and the corresponding % fluorescence quenching value was given prior to correction for the inner filter effect.

PT preparation	Kd (μ M)	fluorescence quenching, %	χ^2
Mutant	63	23	0.54
Native	52	36	1.72
Native (Lobban <i>et al.</i> , 1991*)	37	~60	-
Mutant, 'activated'	21	44	1.53

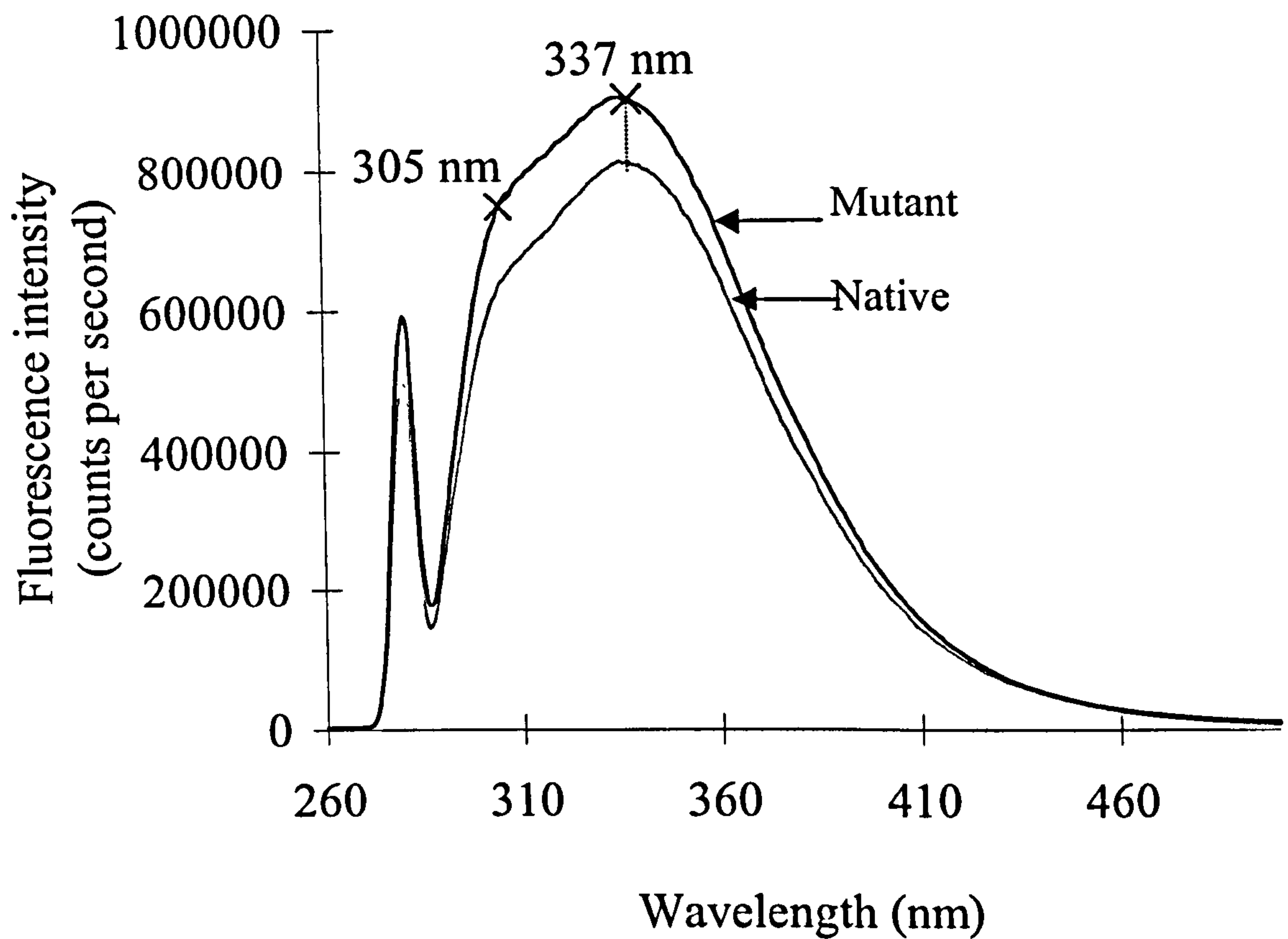


Figure 3.1 Fluorescence of native and mutant PT

Fluorescence spectra of native and mutant pertussis toxin (PT) ($\sim 30 \mu\text{g/mL}$) in PBS, pH 7.4. An excitation λ of 280 nm was used with excitation and emission band passes of 4.25 and 4.5 nm respectively.

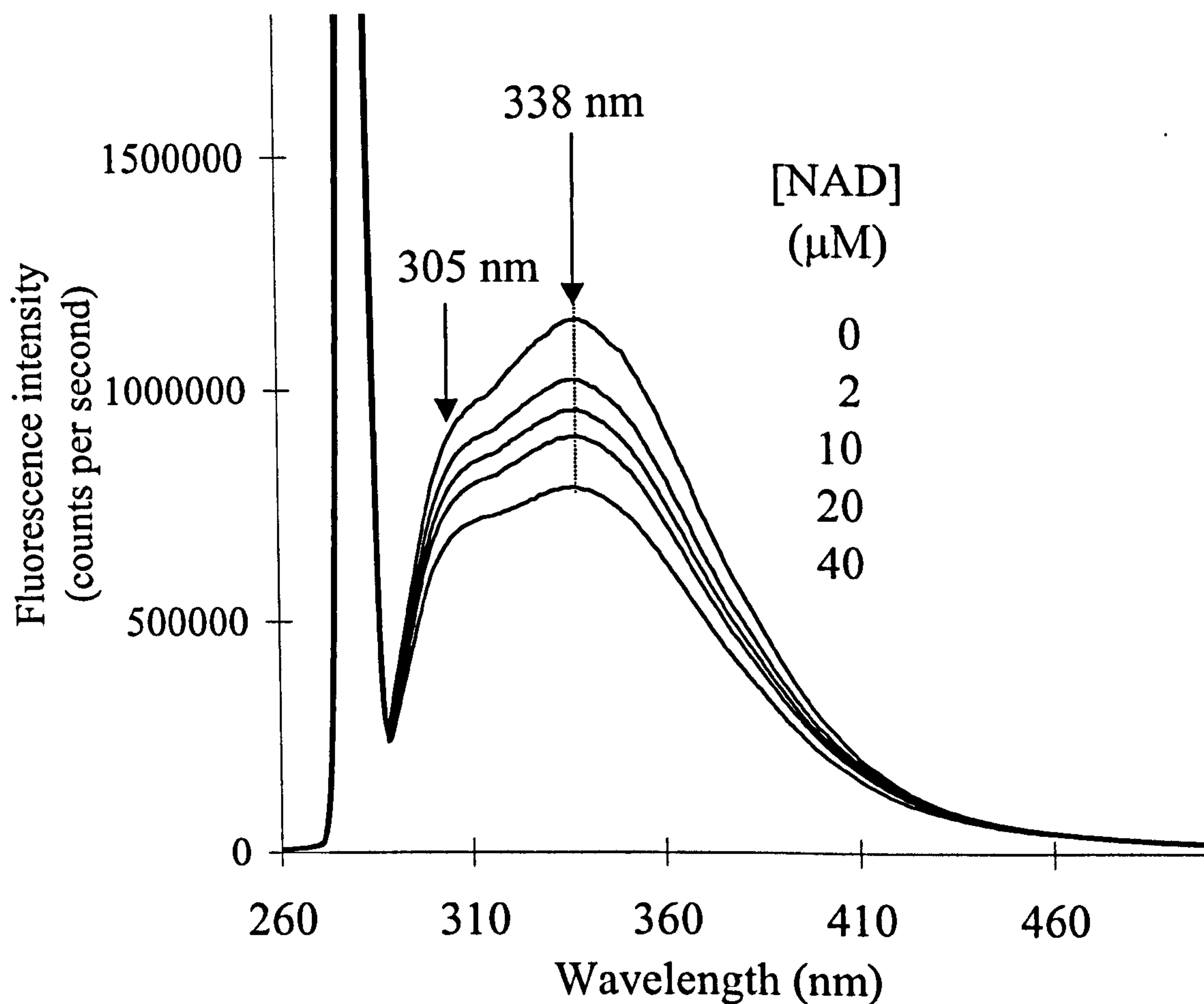


Figure 3.2 (A) Quenching of fluorescence by NAD binding to PT
 Fluorescence spectra of mutant pertussis toxin (PT) before and after the addition of NAD^+ , 0 - 40 $\mu\text{g/mL}$. The concentration of PT was 50 $\mu\text{g/mL}$ in PBS pH 7.4, an excitation λ of 280 nm was used and excitation and emission band passes were set at 4.25 and 4.5 nm respectively. The full quenching data for mutant PT is presented as fluorescence intensity maximum against NAD concentration in Figure 3.2 (B).

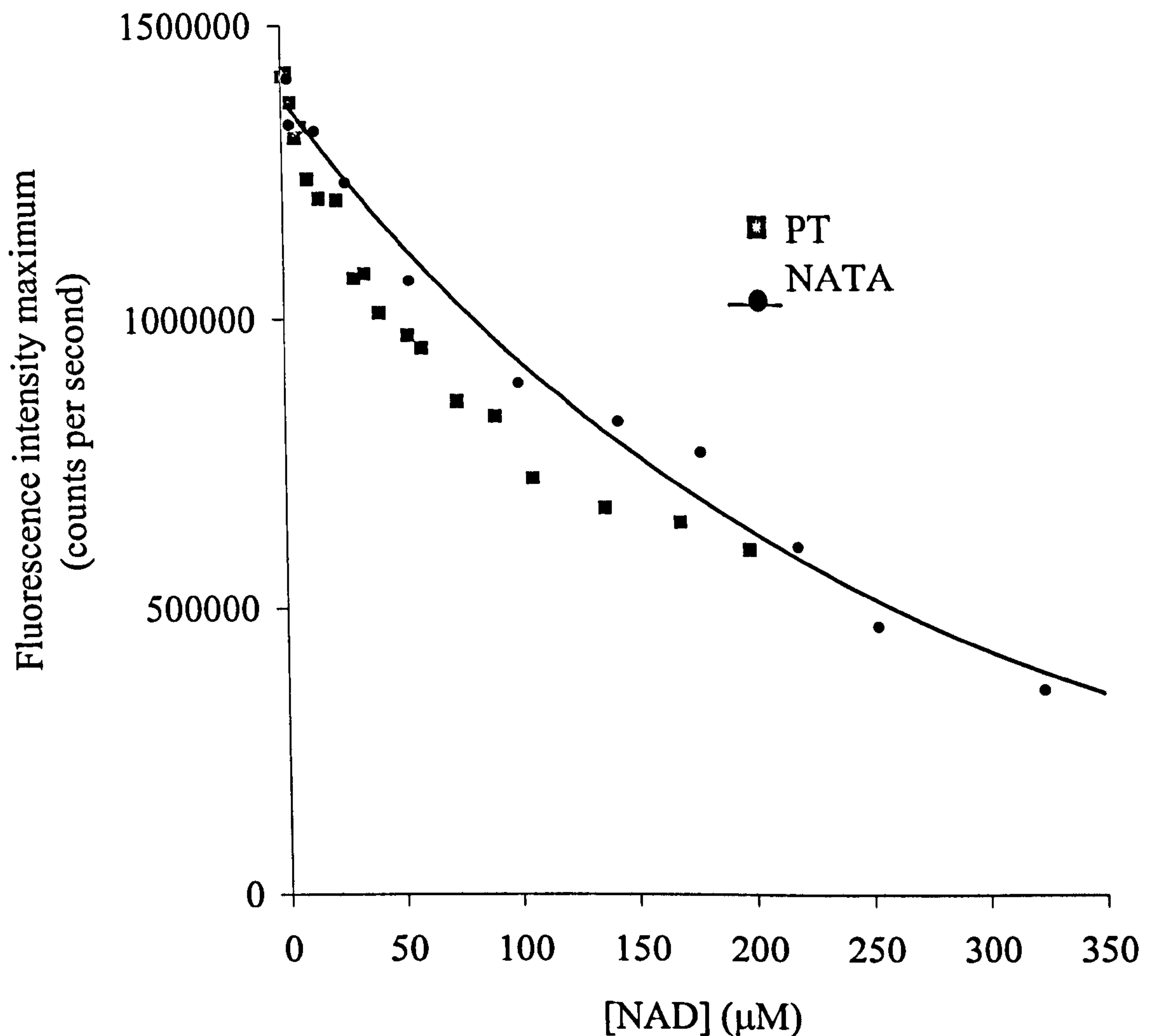


Figure 3.2 (B) Quenching of fluorescence by NAD binding to PT:
Titration curve

Comparison of fluorescence quenching of mutant pertussis toxin (PT) and the tryptophan derivative N-acetyl tryptophanamide (NATA), with the increasing addition of NAD^+ (0 - 300 mM). In separate titrations, the maximum fluorescence intensity of PT or NATA was measured on each addition of NAD. NATA fluoresces but does not bind to NAD, and therefore was used to correct the PT titration data for the non-specific quenching of fluorescence due to absorbance of the exciting light by NAD (inner filter effect). The PT fluorescence intensities were corrected (not shown) for the reduction observed in NATA fluorescence, which is due to the inner filter effect of NAD. The dissociation constants (K_d) were calculated from the corrected titration data for native, mutant and 'activated' mutant PT, using the EquFit5 programme and are presented in Table 3.1.

3.2.1.2 Analysis of 'activated' and NAD⁺-bound 'activated' mutant PT fluorescence

For 'activation', mutant PT was treated with 100 mM dithiothreitol (DTT) at room temperature for two hours and the solution was extensively dialysed. Free sulphhydryl was quantified immediately after 'activation', and also after storage for one month at 4 °C, by the addition of Ellman's reagent and the measurement of absorbance by 2-nitro-5-thiobenzoate at 412 nm.

Intrinsic fluorescence spectroscopy was used, as described above (section 3.2.1.1), to analyse non-'activated' mutant PT, 'activated' mutant PT, 'activated' mutant PT after storage for one month at 4 °C and 'activated' mutant PT with NAD⁺ added. The K_d values for NAD⁺ binding were also determined for mutant PT after 'activation', by measuring intrinsic fluorescence intensity with increasing NAD⁺ concentration as described above (section 3.2.1.1).

Analysis of 'activated' mutant PT by fluorescence

After reduction, or 'activation', of mutant PT with 100 mM DTT for two hours at room temperature, 2.2 moles of free sulphhydryl per mole of PT were detected (Table 3.2). This indicated that one out of a total of 13 disulphide bonds in the mutant PT molecule was broken. The reduction of this disulphide bond corresponded with a decrease in the K_d of mutant PT for NAD⁺, from a K_d of 63 to 21 µM (Table 3.1). These results implicated the cleaved disulphide bond as that of Cys-41 – Cys-201, in the putative active site.

Table 3.2 Disulphide bond cleavage by ‘activation’ of mutant PT

DTNB (5,5'-dithiobis(2-nitrobenzoic acid), or Ellman's reagent) liberates one mole of 2-nitro-5-thiobenzoate ($A_{412\text{nm}}$) from reaction with one mole of free sulphhydryl. On addition of DTNB, the absorbance ($A_{412\text{nm}}$) of mutant pertussis toxin (PT) reduced with dithiothrietol (DTT) ('activated'), 'activated' mutant PT after storage for 1 month and PT not reduced (negative control) was measured.. Reduced mutant PT was also examined for absorbance at 412 nm without the addition of DTNB, as an additional control. The molar concentration of 2-nitro-5-thiobenzoate, was calculated according to the Beer-Lambert law and expressed as moles of free sulphhydryl per mole of mutant PT.

Mutant PT preparation	Absorbance at 412 nm	Moles of free sulphhydryl per mole of mutant PT
not reduced + DTNB	0.000	0
reduced	0.000	0
reduced, 1 month storage, +DTNB	0.000	0
reduced + DTNB	0.022	2.19

Concomitant with the disulphide bond cleavage was a significant decrease in the band density for S1, relative to the other subunit bands, when 'activated' PT was examined by SDS-PAGE under non-reducing conditions (data not shown). Further evidence implying the S1 active site Cys-41 – Cys-201 as the cleaved disulphide bond, was that the concentration of DTT in this experiment was of the same order of magnitude as that necessary to produce almost full catalytic activity of native PT in vitro (Moss *et al.*, 1983).

The 'activation' of PT was accompanied by a 2 nm red-shift in Trp fluorescence from 338 to 340 nm (Figure 3.3). This suggested that cleavage of the Cys-41 – Cys-201 disulphide bond promoted a structural modification causing exposure of nearby Trp to a more polar environment. The Trp-26 indole ring is located only 3.85 Å from the Cys-41 – Cys-201 disulphide bond, suggesting that the reorientation of this Trp was responsible for the red-shift induced by 'activation'. The reduction appeared to be reversible since after storing the 'activated' mutant PT sample for one month at 4 °C, the free sulphydryls were no longer detectable (Table 3.1), the density of its S1 band in SDS-PAGE was comparable with that of non-'activated' PT (not shown), and the F_{max} was reversed back to 337 nm (Figure 3.3).

Analysis of NAD⁺-bound 'activated' mutant PT by fluorescence

On addition of NAD⁺ to 'activated' mutant PT a 2 nm blue-shift in fluorescence from 340 to 338 nm (Figure 3.3) was observed. This is in contrast to the unchanged F_{max} observed with addition of NAD⁺ to non-'activated' mutant PT (Figure 3.2A). These results suggest

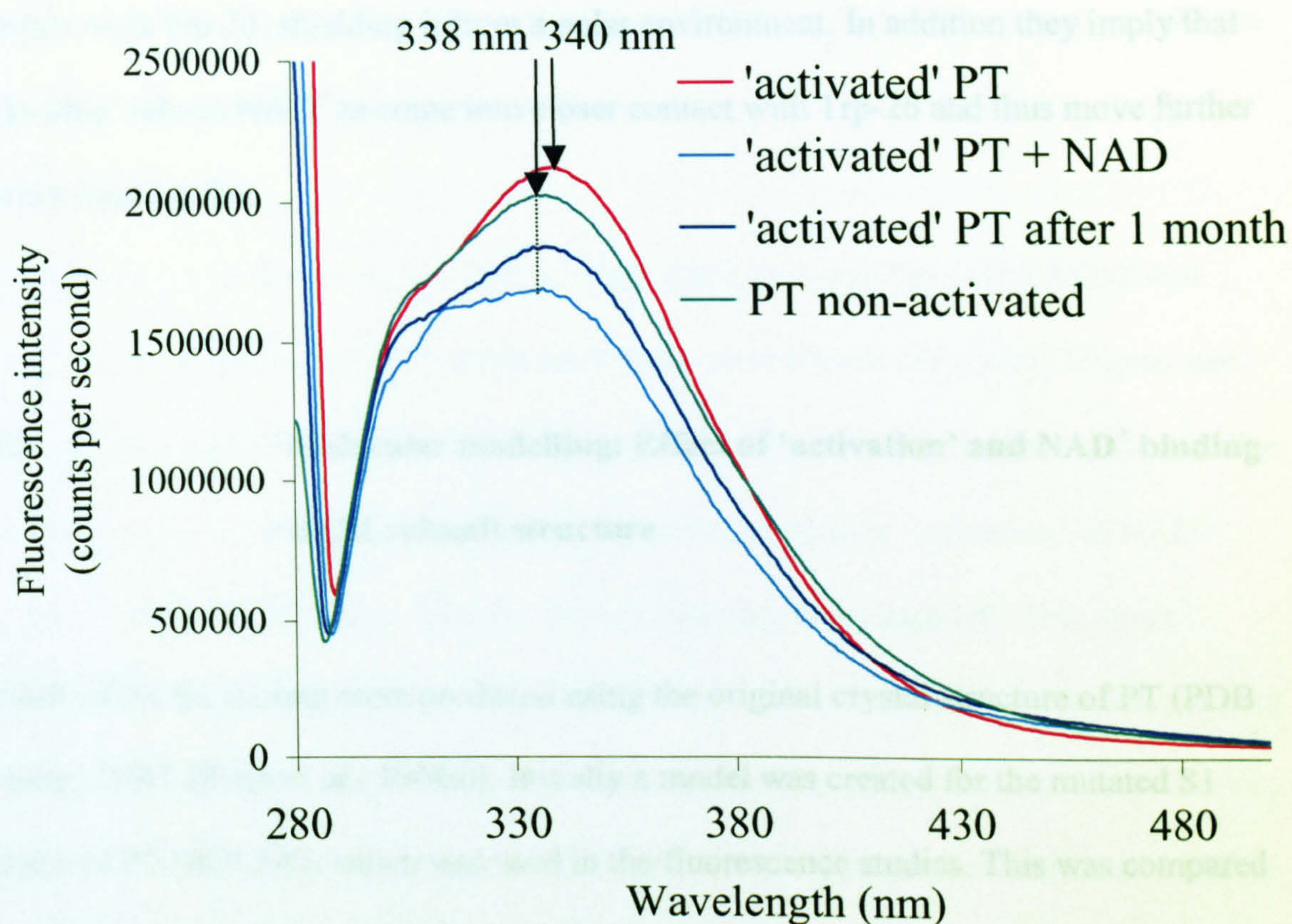


Figure 3.3 Effect of 'activation' and NAD binding on fluorescence spectrum

Fluorescence spectra of non-activated pertussis toxin (PT), 'activated' PT, 'activated' PT after one month storage at 4 °C and 'activated' PT after addition of NAD (50 μ M). The colours of the fluorescence spectra match those of the corresponding molecular models. The concentration of PT was 50 μ g/mL, the excitation λ used was 280 nm and excitation and emission band passes were set at 4.25 and 4.5 nm respectively.

that NAD^+ causes a structural change in 'activated' mutant PT near Trp 26 and/or interacts with Trp 26, shielding it from a polar environment. In addition they imply that 'activation' allows NAD^+ to come into closer contact with Trp-26 and thus move further into the binding site.

3.2.2 Molecular modelling: Effect of 'activation' and NAD^+ binding on S1 subunit structure

Models of the S1 subunit were produced using the original crystal structure of PT (PDB number, 1PRT (Stein *et al.*, 1994a)). Initially a model was created for the mutated S1 subunit of PT-9K/129G, which was used in the fluorescence studies. This was compared with the S1 model of native sequence to identify possible structural differences.

Secondly, in the majority of the study, molecular mechanics simulations were performed on the S1 subunit of native sequence to produce three models: non-'activated', 'activated' and NAD^+ -bound 'activated', and their structures were analysed in detail.

3.2.2.1 Comparison of mutant and native S1 subunit molecular models

The majority of the molecular modelling studies were performed on the S1 model of native sequence from the original crystal structure for PT, and compared with fluorescence the studies of mutant PT. Therefore, a mutant S1 model was generated and

compared with the original S1 model to identify any possible structural differences.

Mutation of the S1 subunit was simulated in insightII by changing Arg-9 to Lys and Glu-129 to Gly followed by energy minimisation.

Superimposition of the native and mutant (Arg-9 to Lys, Glu-129 to Gly) S1 subunit models demonstrated that their peptide backbones were almost completely aligned and that only small rotations in the ϕ , ψ and χ_1 angles occurred (data not shown). Most of the rotations were not significant compared to those observed after 'activation' or NAD⁺ binding, with the exception of Thr-53, which rotated by more than 20°. This result suggests that, in combination with the mutation of Arg-9 and Glu-129, the accompanying structural change in the catalytically important residue Thr-53 may further contribute to the catalytic inactivation of this mutant. Other than these changes, the mutant and native S1 models were similar. The following modelling experiments were performed on the native S1 subunit molecule, of the original crystal structure.

3.2.2.2 Analysis of 'activated' and NAD⁺-bound S1 subunit by molecular modelling

From the original crystal structure of PT (PDB number, 1PRT Stein *et al.*, 1994a) three models of the S1 subunit were produced, and are presented: non-'activated' S1, 'activated' S1 and NAD⁺-bound 'activated' S1. The S1 subunit was 'activated', by breaking and protonating the Cys-41 – Cys-201 disulphide followed by energy

minimisation. In order to dock NAD^+ into the 'activated' S1 molecule, distances measured between NAD^+ and homologous amino acids (Table 3.3) in the DT- NAD^+ complex (1TOX, Bell and Eisenberg, 1996) were applied as restraints during energy minimisation. The non-'activated' S1, 'activated' S1 and NAD^+ -bound 'activated' S1 molecules were minimised using the AMBER force field and software (Cornell *et al.*, 1995) allowing flexibility in both the whole protein molecule and in NAD^+ . Rotation of the S1 dihedral bond angles, ϕ (defined by atoms $\text{N}' - \text{C} - \text{C}\alpha - \text{N}$), ψ (defined by atoms $\text{C} - \text{C}\alpha - \text{N} - \text{C}'$) and χ_1 (defined by atoms $\text{N} - \text{C}\alpha - \text{C}\beta - \text{C}\gamma$ etc) (measured in insightII), and change in percentage solvent accessibility of amino acid side chains (determined using NACCESS, (Hubbard and Thornton, 1993)) were calculated both after 'activation' of the S1 molecule and NAD^+ binding to the 'activated' model.

Analysis of the 'activated' S1 subunit molecular model

By cleaving the Cys-41 – Cys-201 disulphide bond ('activation'), there was significant movement of the Cys-201 and, more markedly, Cys-41 side chains (Figures 3.4A and 3.4B). This movement, along with a significant rotation of the Trp-26 ψ angle by 15° , positioned the indole ring of Trp-26 in much closer proximity to the Cys-41 residue. A 1.9 Å shift in the $\text{C}\alpha$ atom of Trp-26 was measured. The indole ring shifted from a position 3.85 Å from the Cys-41 S – S bond before 'activation', to 2.03 Å from the newly formed polar S – H bond after 'activation'. This proximity to a polar group could account for the red-shift in Trp fluorescence maximum observed after reduction (Figure 3.3). Additionally there was movement of α -helix amino acids 185 – 210 (Figure 3.4C), some becoming more solvent accessible and others more buried (Figure 3.4D).

Table 3.3 Homologous amino acids of PT and DT

Homologous amino acids of pertussis toxin (PT) and diphtheria toxin (DT) obtained from sequence alignments of the structurally equivalent residues of pertussis toxin (PT), diphtheria toxin (DT), *Escherichia coli* heat-labile enterotoxin (LT) and *Pseudomonas aeruginosa* exotoxin-A (PAETA), taken from (Stein, 1994).

PT	Tyr-8	Gly-86	Glu-129	Tyr-130	Val-91
DT	Tyr-20	Gly-78	Glu-148	Tyr-149	Val-83

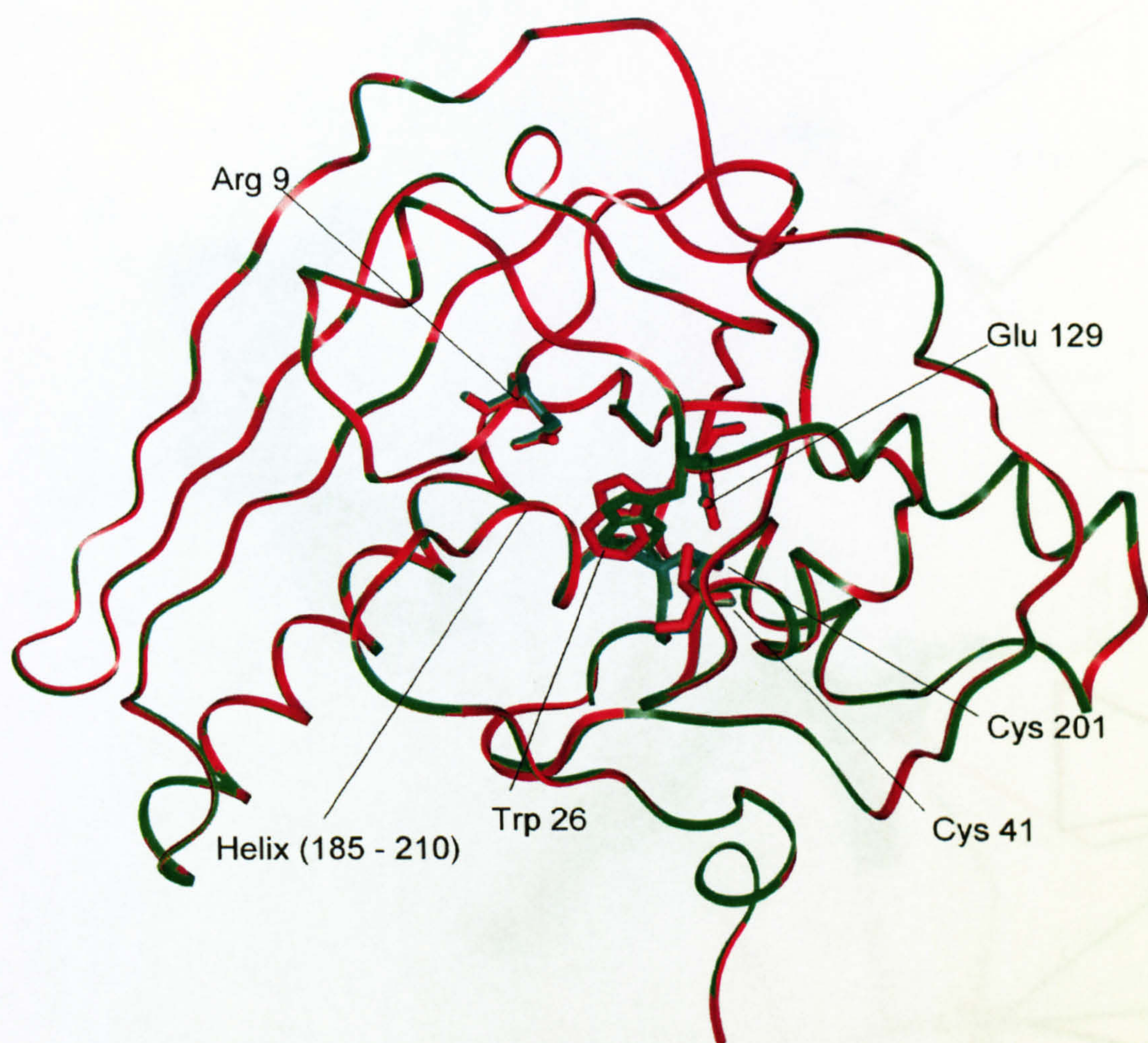


Figure 3.4 (A) 'Activated' S1 subunit model

Superimposed models of the S1 subunit before and after 'activation', by cleavage of the Cys-41 – Cys-201 disulphide bond. Model minimised using AMBER software.

Non-'activated' S1 subunit – green; 'activated' S1 subunit – red.



Figure 3.4 (B) 'Activated' S1 subunit model (enlarged)

Superimposed enlarged sections of the S1 subunit models before and after 'activation', by cleavage of the Cys-41 – Cys-201 disulphide bond.

Non-'activated' S1 subunit – green; 'activated' S1 subunit – red.

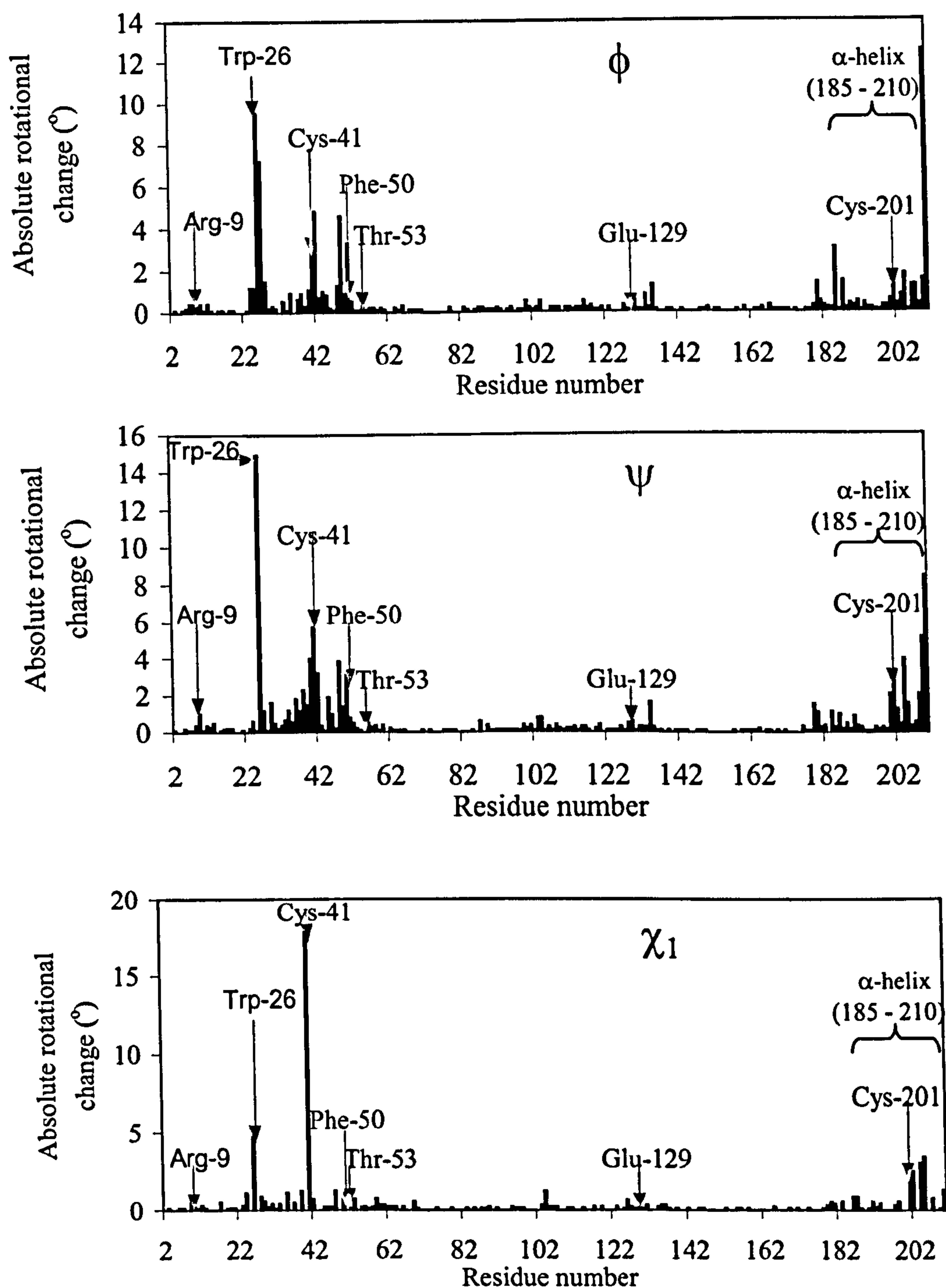


Figure 3.4 (C) 'Activated' S1 subunit, bond rotations
 Change in PT amino acid dihedral bond angles (ϕ , ψ and χ_1) after 'activation'. The bond angles for the 'activated' and NAD-bound 'activated' PT models were measured in insightII and the smallest absolute difference between them was calculated.

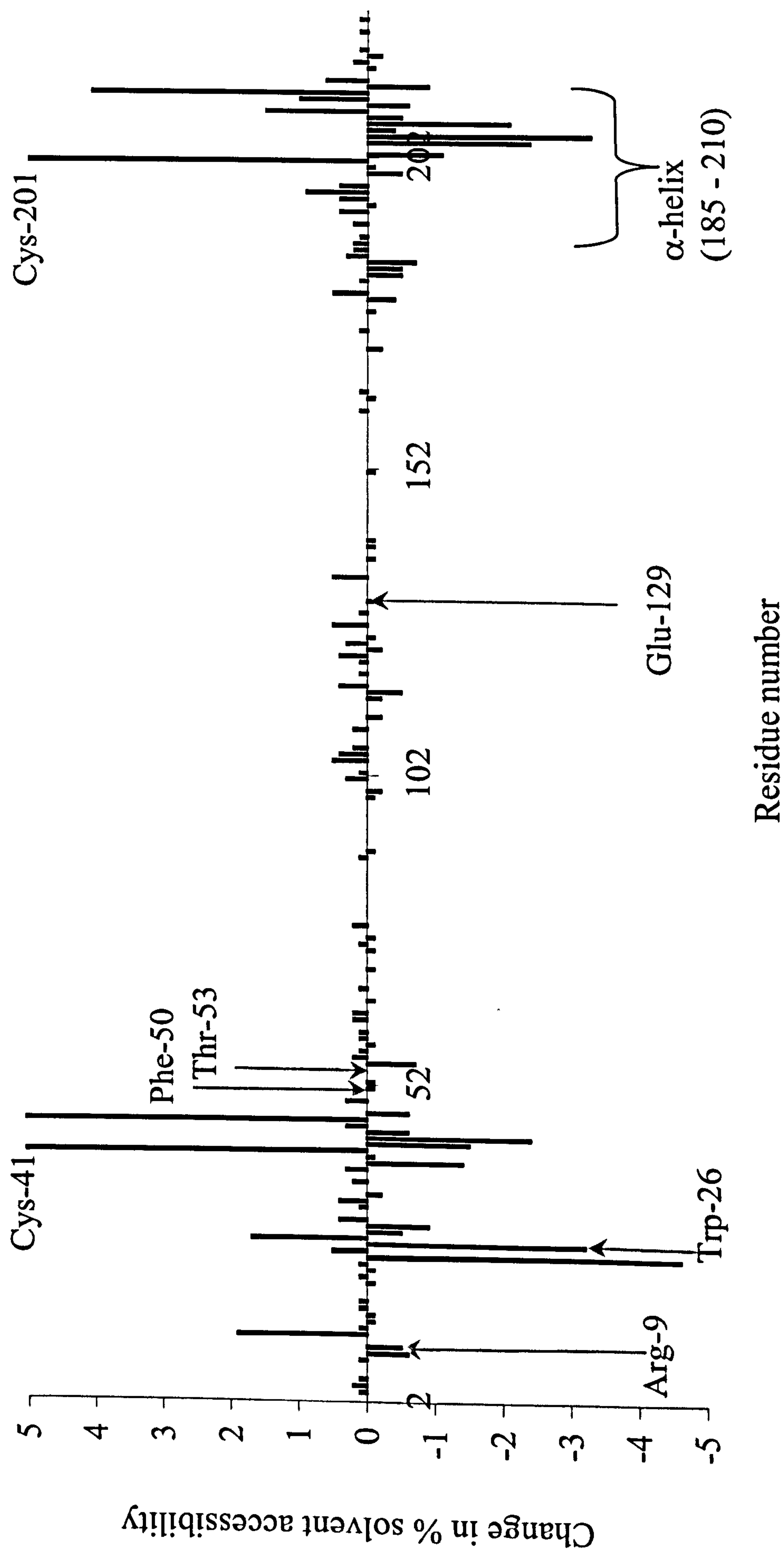


Figure 3.4 (D) 'Activated' S1 subunit, amino acid accessibility
 Change in solvent accessibility after 'activation'. The % solvent accessibilities for the amino acid side chains of non-'activated' and 'activated' PT models were calculated using the NACCESS programme (Hubbard and Thornton, 1993). The difference in accessibility after 'activation' was calculated.

Analysis of NAD⁺-bound 'activated' S1 subunit molecular model

Trp-26 exists on a turn in a helix, and is partially solvent exposed, being 37 % accessible to solvent, as determined by a solvent accessibility programme (NACCESS, (Hubbard and Thornton, 1993)). On NAD⁺ binding, Trp-26 moves further into the molecule, into a less solvent accessible position by a 100° rotation of its side chain (3.5 Å shift in the indole ring), compared with the average χ_1 rotation for all side chains of under 10° (Figures 3.5A – C). Again this is in agreement with the blue-shift observed in intrinsic fluorescence spectroscopy upon addition of NAD⁺.

The side chains of active site residues such as Tyr-8, Arg- 9 and Glu-129 also rotate significantly (Figure 3.5B) and their close proximity to the NAD⁺ molecule in the model was observed (Figure 3.5A). Other catalytically important residues, Phe-50 and Thr-53, did not appear to move significantly on NAD⁺ binding but the peptide backbone of the two residues between them, Val-51 and Ser-52, did move considerably. Further movement of α -helix amino acids 185 – 210 also occurred on NAD⁺ binding (Figure 3.5B) and the α -helix moved out of the binding site (Figure 3.5A) into a more solvent accessible position, exposing the G-protein binding residues (195 – 204; Figure 3.5D).

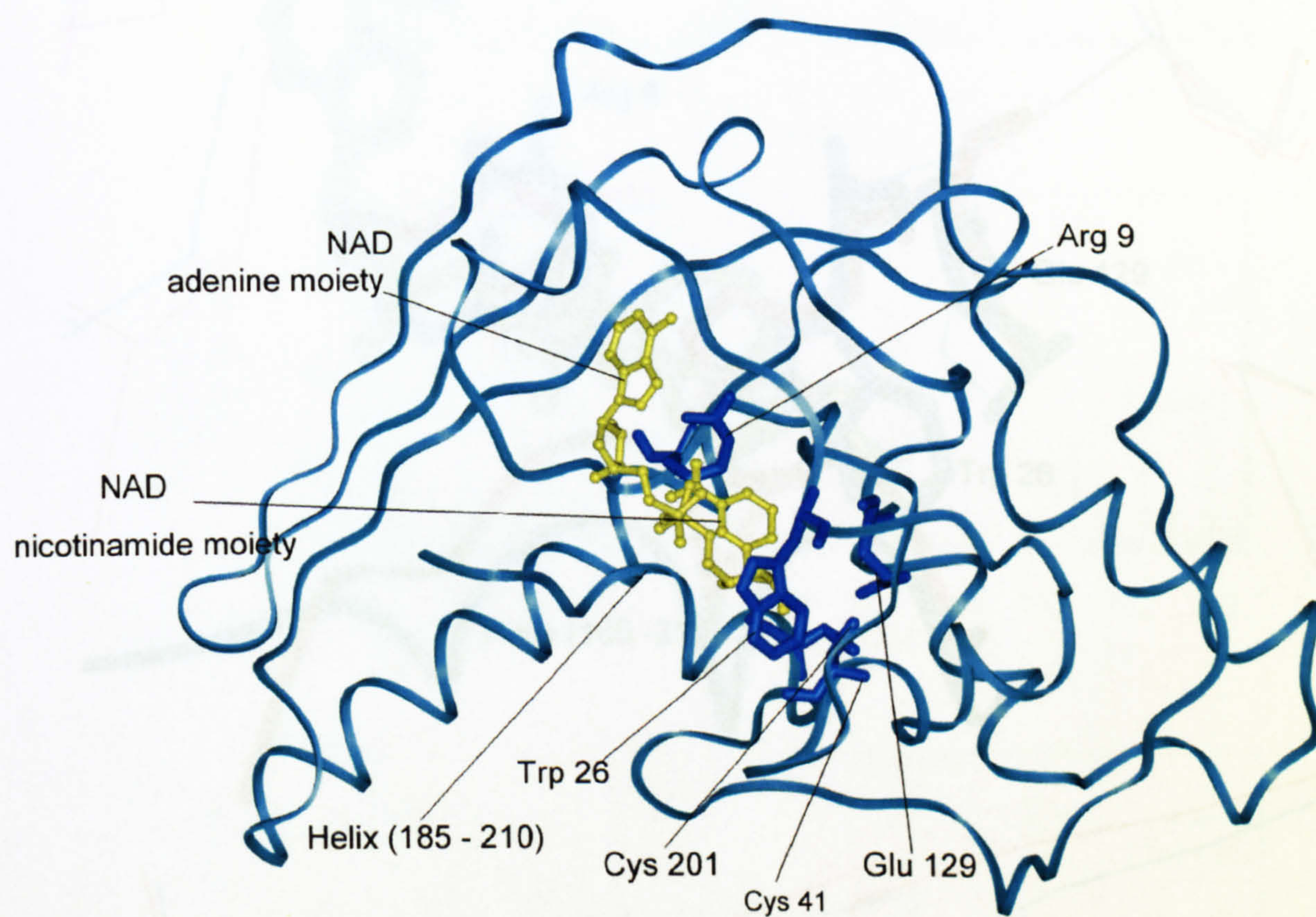


Figure 3.5 (A) NAD-bound 'activated' S1 subunit model

Model of the 'activated' S1 subunit after NAD binding. Model minimised using AMBER software.

NAD-bound S1 subunit – blue; NAD – yellow.

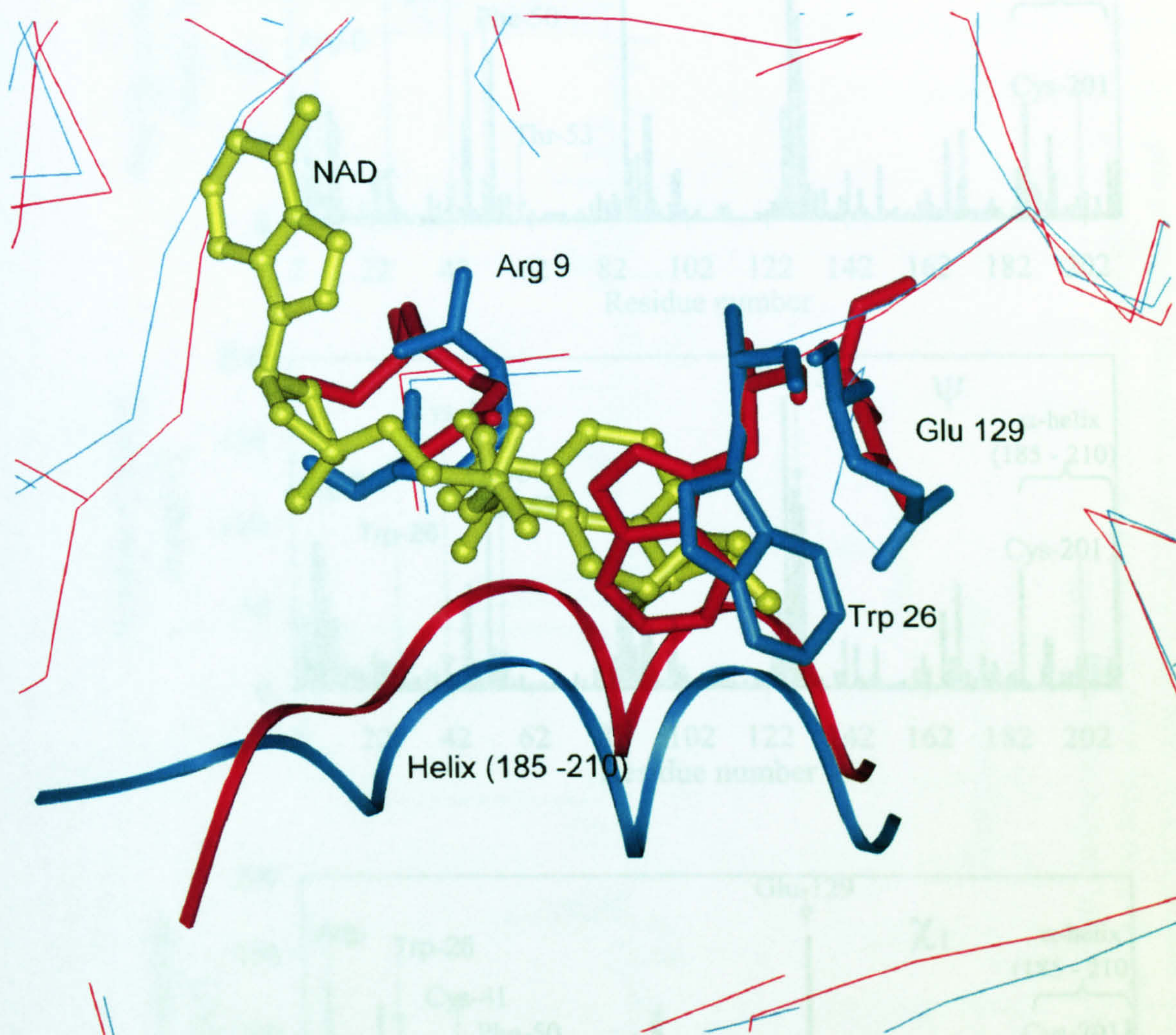


Figure 3.5 (B) NAD-bound 'activated' S1 subunit model (enlarged)

Superimposed enlarged sections of the 'activated' S1 subunit models before and after NAD binding.

'Activated' S1 subunit before NAD binding – red; NAD-bound 'activated' S1 subunit – blue; NAD – yellow.

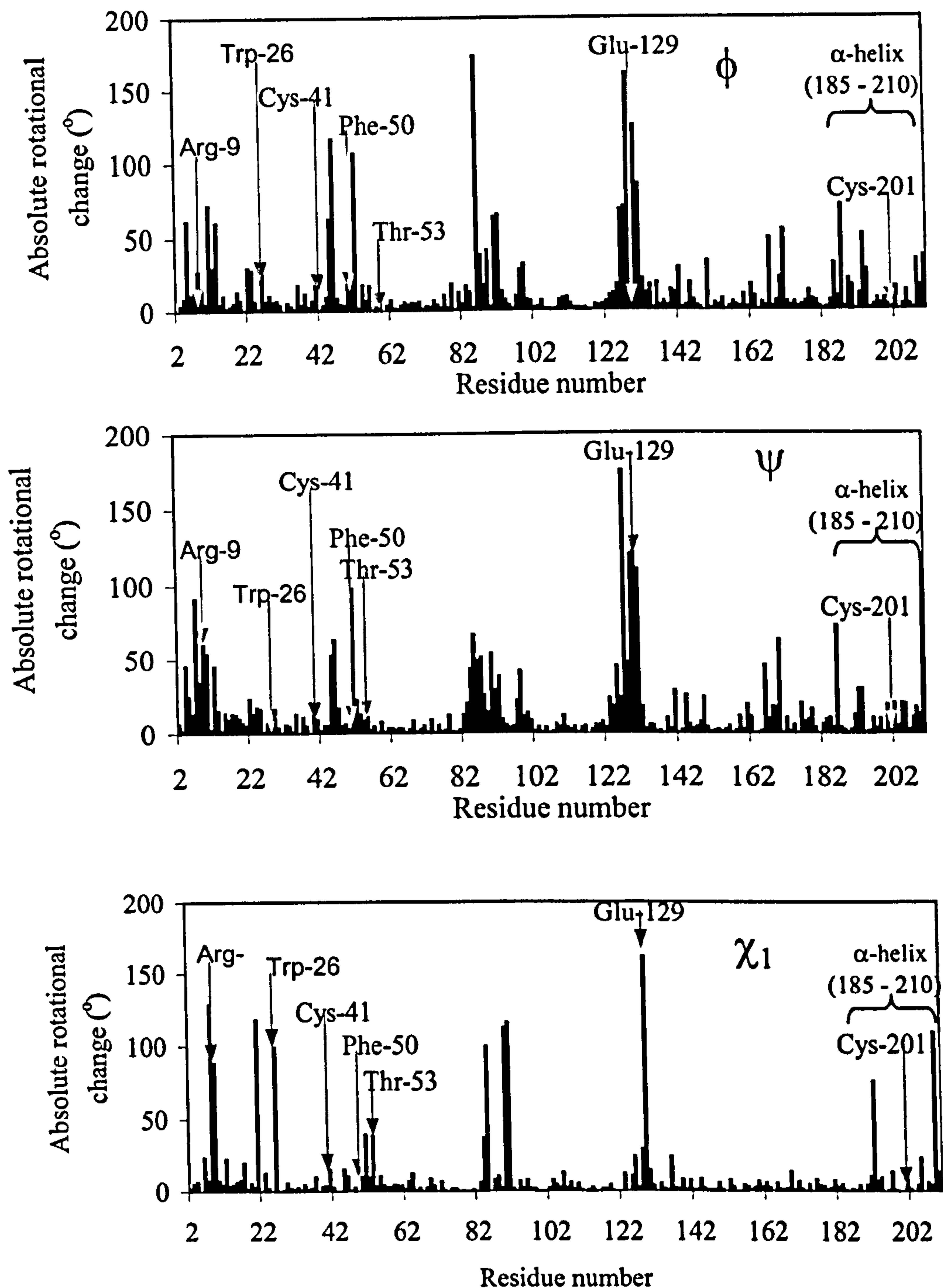


Figure 3.5 (C) NAD-bound 'activated' S1 subunit, bond rotations
 Change in PT amino acid dihedral bond angles (ϕ , ψ and χ_1) after NAD binding. The bond angles for the non-'activated' and 'activated' PT models were measured in insightII and the smallest absolute difference between them was calculated.

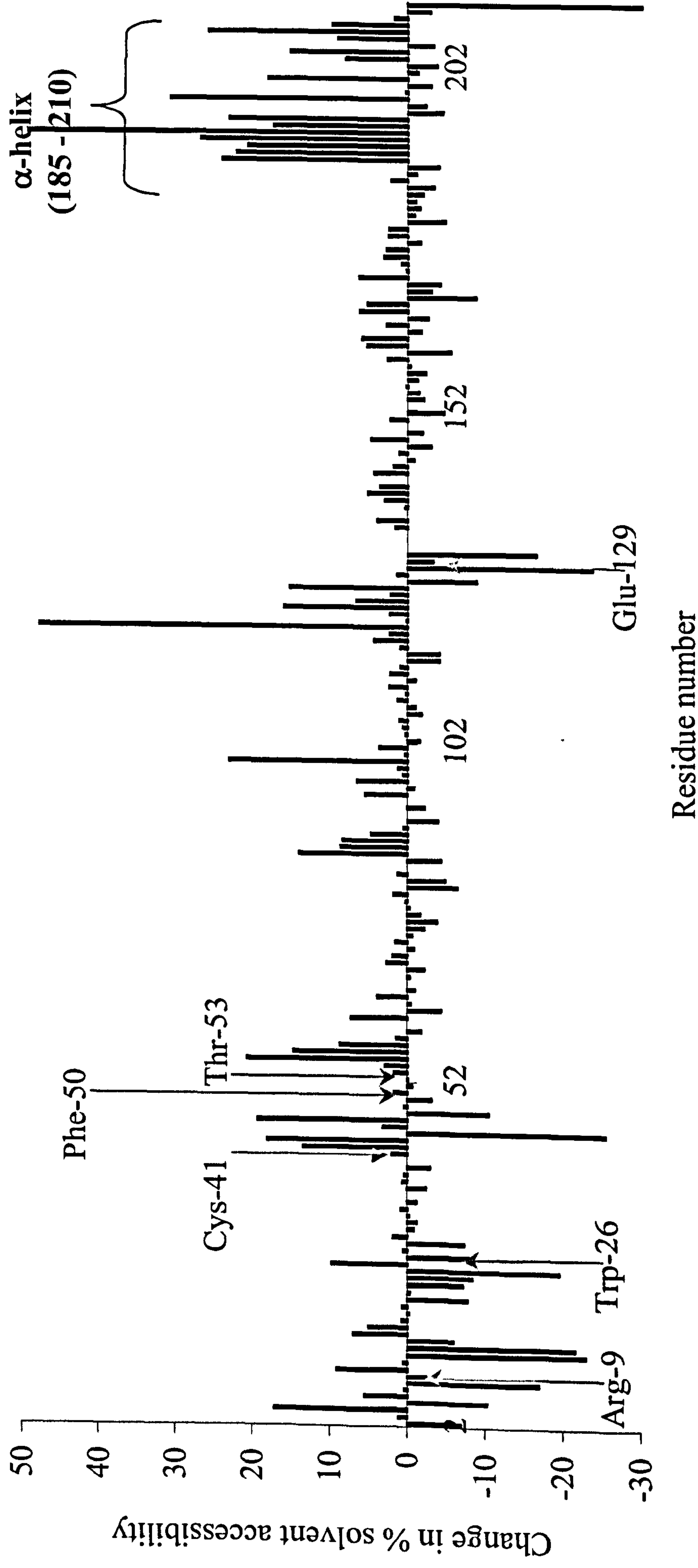


Figure 3.5 (D) NAD-bound 'activated' S1 subunit, amino acid accessibility
 Change in solvent accessibility after NAD binding. The % solvent accessibilities for the amino acid side chains of 'activated' and NAD-bound 'activated' PT models were calculated using the NACCESS programme (Hubbard and Thornton, 1993). The difference in accessibility after NAD binding was calculated. The amino acids comprising the G-protein binding site are 195 - 204.

3.3 Discussion

Many assumptions have previously been made about the binding of NAD⁺ to PT, from comparisons with other structurally and functionally homologous bacterial toxins (Domenighini *et al.*, 1991). The present investigation has revealed models for both the 'activated' and NAD⁺-bound forms of the S1 subunit, which are wholly supported by results from fluorescence spectroscopy. These models also bear out some previous predictions and agree with active site mutagenesis experiments.

Until now evidence for the physical requirements of catalytic activation has been speculative. From the original crystal structure of non-'activated' PT (Stein *et al.*, 1994a), it was envisaged that 'activation' or cleavage of the active site disulphide bond, Cys-41 – Cys-201, would be required to allow the NAD⁺ binding site to open up by movement of the amino acid sequence 184 – 203 (Stein *et al.*, 1994a). The 'activated' computer model of S1 from this study illustrates that significant structural adjustment did occur within an α -helical region defined by amino acids 185 – 210. This model further illustrates movement of the catalytically important Trp-26 residue into the binding site, to a position close to the S – H bond of Cys-41, and this is supported by a red shift in intrinsic fluorescence spectroscopy of mutant PT upon reduction with DTT. It appears that 'activation' by cleavage of Cys-41 – Cys-201 is required to release the α -helix (residues 185 – 210) and orient the active site residues in preparation for subsequent NAD⁺ binding and catalysis.

The novel 'activated' NAD⁺-bound PT model demonstrated that NAD⁺ docked into the putative binding site. This is a cleft defined by a motif composed of an α -helix and a β -strand facing a large loop, which had been previously been predicted by homology studies of PT, LT1 and LT2, CT, PAETA and DT (Domenighini *et al.*, 1991; Domenighini *et al.*, 1994). Involvement of catalytically important residues, Tyr-8, Arg-9, Phe-50, Thr-53, Glu-129 and Trp-26 previously identified in mutagenesis experiments (Pizza *et al.*, 1988), was also clearly illustrated by the model, as shown by significant rotation of ϕ , ψ and χ_1 angles upon NAD⁺ binding.

The α -helix (185 – 210), observed to adjust upon 'activation', moved significantly out of the active site cleft upon NAD⁺ binding. This model bears out the previous predictions of P. Stein and colleagues (Stein *et al.*, 1994a), that this would be a physical requirement for NAD binding. It is fascinating that movement of the helix not only facilitates NAD⁺ binding but concomitantly reveals a G-protein substrate binding site (Figure 3.5D). The moving α -helix in this model is the predicted 'active site loop' of PT, and homologous such 'active site loops' have also been identified in LT, CT and DT (Locht and Antoine, 1999). Attempts to model the active site using the crystal structure for PT have previously been carried out using only sections of the S1 subunit, within the amino acid sequence 2 – 189, and the separated adenine and nicotinamide moieties of NAD⁺ (Scheuring, Berti, and Schramm, 1998; Cummings, Hart, and Read, 1998). Moreover, in these studies rigid docking schemes were employed (Scheuring, Berti, and Schramm, 1998). Therefore, these studies could not address the involvement of the α -helix occluding the active site.

The modelling studies also revealed that, upon NAD⁺ binding, Trp-26 moved to an even more buried position close to the nicotinamide moiety of NAD⁺. This was in agreement with the blue-shift in fluorescence observed upon addition of NAD⁺. Studies using fluorescence have previously failed to detect such a blue-shift (Seabrook, Atkinson, and Irons, 1991) due to the fact that non-‘activated’ PT was used. Results from the current study do corroborate findings of Cortina and Barbieri (Cortina and Barbieri, 1989) that Trp-26 is involved in NAD⁺ binding. This group also established the importance of an aromatic residue at this position for NAD⁺ binding.

In addition to the blue-shift in PT fluorescence on NAD⁺ binding, fluorescence quenching was concomitantly observed. Past investigations of ADP-ribosylating toxins have highlighted several possible mechanisms for Trp fluorescence quenching with NAD⁺, including base-stacking of the indole ring with the nicotinamide or adenine moieties of NAD⁺ (Beattie, Prentice, and Merrill, 1996; Brandhuber *et al.*, 1988). Aromatic stacking between nucleotides and the indole ring of Trp has been previously recognised as a common mechanism for protein-nucleotide binding (Nishikawa *et al.*, 1988; Khamis *et al.*, 1987; Casas-Finet *et al.*, 1988). However, our model for NAD⁺ binding does not support the mechanism of aromatic stacking of the Trp-26 indole ring. This may be partly due to the fact that the AMBER programme used for minimisation has no explicit force field term for aromatic stacking interactions. However, in agreement with our results, Cortina and Barbieri (1989) demonstrated that ADP-ribosyl transferase activity of PT was not fully maintained with any of the aromatic substitutions of Trp-26, and advocated that

the interaction between NAD^+ and Trp-26 is more involved than aromatic base-stacking alone.

An alternative explanation for the observed quenching of Trp-26 fluorescence is by an electron transfer mechanism, which has also been previously advocated in the fluorescence quenching of Trp-558 of PAETA (Beattie, Prentice, and Merrill, 1996). In the current NAD^+ -bound model of S1, the proximity of NAD^+ to Trp-26 (nicotinamide ribose moiety of NAD^+ is positioned only 2.45 Å from the indole ring of Trp-26) may account for fluorescence quenching by electron transfer.

The role of the active site Trp-26 of PT in the ADP-ribosyl transferase reaction is not yet known. Specific fluorescence quenching of active site Trps upon NAD^+ binding has been found for several protein toxins including PAETA (Trp-558) and DT (Trp-50 and Trp-153; (Beattie, Prentice, and Merrill, 1996; Wilson *et al.*, 1994; Lory *et al.*, 1980). These results suggested that these Trp residues have a specific role in NAD^+ binding. An alternative role for Trp residues in the assimilation of NAD^+ may be in stabilising the hydrophobic NAD^+ -binding cavity, as found for Trp-350 of *Bacillus*-produced vegetative insecticidal protein-2 (VIP2) (Han *et al.*, 1999).

The 'activated' NAD^+ -bound model for PT may form the basis for further research on the mechanism of ADP-ribosylation, common to a variety of bacterial protein toxins. The structural involvement of disulphide bond reduction and NAD^+ binding in the action of PT, may be developed to explain the sequence of events following PT binding that lead to

catalysis *in vivo* including, vesicular trafficking, internalisation and G-protein targeting. The distinct roles of the A- and B-oligomer in the numerous biological properties of PT, including toxic and immunogenic properties, still require clarification. The use of toxins such as PT, CT and LT as vaccines, adjuvants and therapeutic products will depend on further such structural and functional investigation.

4.1**Introduction**

Clinical trials have revealed differences in the efficacy of acellular pertussis vaccines produced by different manufacturers (Hewlett and Cherry.J.D., 1997; Decker and Edwards, 2000; Edwards, 1998), and these discrepancies have been attributed to differences such as the study design of the trials and antigenic composition of the vaccines (Hewlett and Cherry.J.D., 1997). PT is the major toxic and immunogenic antigen of acellular pertussis vaccines (Pittman, 1979; Sato *et al.*, 1980; Oda *et al.*, 1984; Trollfors *et al.*, 1995), and manufacturers employ a variety of detoxification protocols which involve different chemical treatments. Variations in the antigenic properties of PT toxoids could be one of the factors which contribute to the observed differences in the clinical efficacy of acellular pertussis vaccines.

To investigate this possibility, five different PT toxoids were obtained from manufacturers of acellular pertussis vaccines for structural and immunological analysis. The toxoids are identified by letters A – E and were each produced under different manufacturing protocols using different toxoiding agents: A – genetically detoxified (PT-9K/129G) with low FA treatment (0.035 %, w/v, FA), B – glutaraldehyde plus formaldehyde treatment, C – glutaraldehyde treatment, D – tetranitromethane treatment,

and E – hydrogen peroxide treatment. The toxoids and the untreated native PT samples from each manufacturer were analysed by SEC/UV and fluorescence spectroscopy, to assess the effects of the different toxoiding procedures on the molecular structure and size of the resultant PT molecules. SDS-PAGE and Western blotting were carried out, using subunit specific monoclonal antibodies (mAbs), in order to investigate subunit cross-linking and epitope modification upon toxoiding. In addition, the relative immunogenicities of the toxoids were determined by assessing the induction of murine anti-PT total IgG by ELISA. IgG1 and IgG2a subclass antibodies were also assayed, as markers for Th2- and Th1-type immune response respectively (Male *et al.*, 1996). Furthermore, the protective efficacy of each toxoid was determined by aerosol challenge in the mouse model.

4.2 Results

4.2.1 The effect of different chemical toxoiding on the structure of PT

The untreated native PT samples obtained from each manufacturer were all analysed by SEC/UV and fluorescence spectroscopy and gave comparable results. Therefore, the results from one native PT sample are presented.

Analysis of PT toxoids by SEC/UV

The five different toxoids, A – E, and representative native PT sample were subject to SEC/UV using an FPLC system equipped with a Superdex 200 column, at a flow rate of 0.5 mL/min in PBS buffer, pH 7.5. The elution of protein was detected both at 214 and 280 nm, and the 214 nm trace is presented (Figure 4.1).

The untreated native PT typically eluted as a single broad peak at 18 mL (Figure 4.1). For toxoid A, most of the protein eluted in an earlier peak at 16.7 mL, which was much broader and had a slight shoulder at about 18 mL. For the other aldehyde-treated toxoids, B and C, and the tetranitromethane-treated toxoid, D, the bulk of material eluted much earlier, starting at ~ 10 mL, as a series of non-uniform peaks. For these toxoids well defined peaks appeared at 12.3 mL for toxoid B, 17.1 mL for toxoid C and 14.9 mL for toxoid D. The hydrogen peroxide-treated toxoid E was quite different from the other toxoids, and eluted as a single peak at 19.4 mL, with a similar profile to the untreated native PT.

The variety of profiles obtained for toxins A – E indicate that different toxoiding chemicals and procedures caused different modifications to the PT molecules, which became associated in different ways. Toxoid A, which is genetically detoxified and treated with 0.035 % (w,v) FA, appeared to contain slightly higher molecular weight material than the untreated native PT. For the formaldehyde plus glutaraldehyde-treated toxoid B, glutaraldehyde-treated toxoid C and tetranitromethane-treated toxoid D, the earlier-eluting, broad, non-uniform spectra suggest these toxoiding chemicals produced

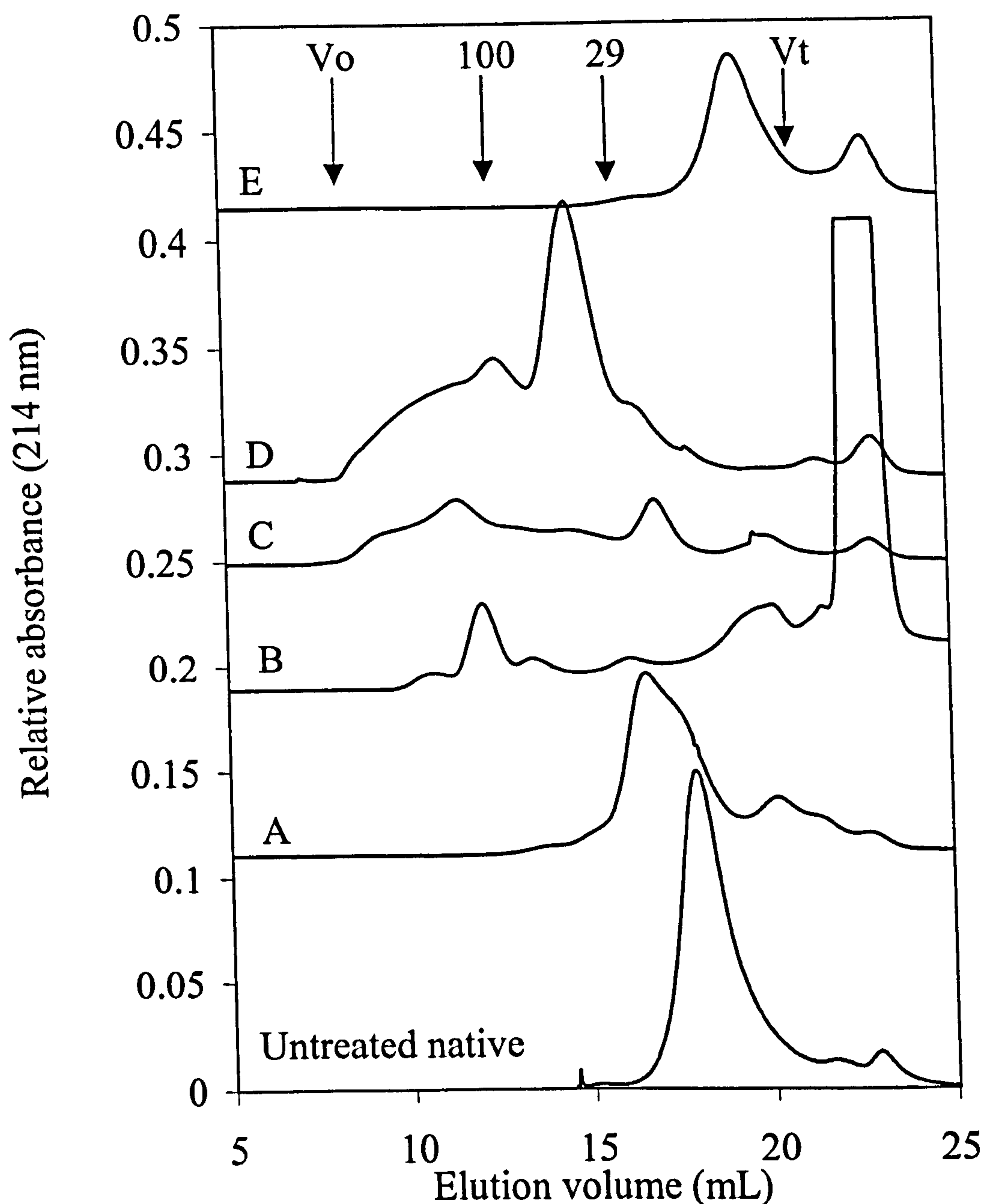


Figure 4.1 Effect of different different toxoiding procedures on the size of PT
 Pertussis toxin (PT) toxoids obtained from five different manufacturers (A - E) and an untreated native PT sample (15 μ g protein in 200 mL PBS, pH 7.5) were analysed using size exclusion chromatography with UV detection (SEC/UV). The FPLC system was equipped with a Superdex 200 column and samples were eluted in PBS, pH7.5. Each manufacturer employed a different PT toxoiding agent: A - genetically detoxified plus low formaldehyde treatment, B - glutaraldehyde plus formaldehyde treatment, C - glutaraldehyde treatment, D - tetranitromethane treatment, E - hydrogen peroxide treatment. Elution positions of selected molecular weights (labelled in kDa) and of the exclusion volume (V_o) and total volume (V_t), as determined by calibration with protein standards, are indicated with arrows. Absorbance at 214 nm is shown.

heterogeneous species of higher molecular weight than the other toxoids. In addition, the early eluting, more defined peaks suggest that there were some high molecular weight molecules that were homogeneous in size. This implies that they may have been created from the favoured associations of holotoxin or oligomeric, dimeric or monomeric subunits. In contrast to the other toxoids, toxoid E eluted with a similar profile to the untreated native PT. Its slightly later elution may suggest that the charge or shape of the PT molecule had been modified to some extent by hydrogen peroxide.

Analysis of PT toxoids by fluorescence spectroscopy

PT contains six Trp residues and 62 Tyr residues, therefore intrinsic fluorescence of these environmentally sensitive aromatic side chains is potentially a sensitive indicator of changes in the molecular structure of PT. The PT toxoids, A – E, were subject to fluorescence spectroscopy by λ_{ex} of 280 nm. The fluorescence was scanned from 260 to 500 nm and the base line fluorescence of the sample buffer (PBS, pH 7.5) was subtracted.

The untreated native PT displayed a typical fluorescence spectra with fluorescence emission wavelength maximum (Fmax) from Trp fluorescence at 337 nm and a shoulder from Tyr fluorescence at ~ 305 nm (Figure 4.2) (Seabrook, Atkinson, and Irons, 1991). Both toxoids A and B displayed a significantly red-shifted Fmax of 340 nm and 342 nm respectively, but retained spectra of a similar shape to that of the untreated native PT (Figure 4.2). Toxoid C produced a fluorescence spectrum which also had a red-shifted Fmax of 345 nm (Figure 4.2). In addition, the intensity of fluorescence was significantly diminished with respect to the untreated native PT preparation, even though the same

concentration of protein was used. Toxoid D also exhibited a red-shifted F_{\max} and, like toxoid C, also gave lower fluorescence intensity than expected (Figure 4.2). The fluorescence of both toxoids C and D, even at three times the concentration ($\sim 90 \mu\text{g/mL}$) of the native PT, resulted in half of the fluorescence intensity (not shown). Toxoid E produced a fluorescence spectrum which displayed a single peak at 305 nm but appeared to be devoid of any contribution from Trp (Figure 4.2).

The very different fluorescence spectra obtained from PT toxoids illustrate that different chemical treatments cause a variety amino acid modifications, leading to an assortment of local conformational changes in the vicinity of aromatic residues and/or specific alterations of the aromatic residues. All of the toxoids, with the exception of toxoid E, displayed a red-shift in F_{\max} which can be indicative of Trp exposure to solvent, suggesting protein unfolding. Therefore, these results suggest that the FA treatment of toxoid A, the glutaraldehyde plus formaldehyde treatment of toxoid B, the glutaraldehyde treatment of toxoid C and the tetranitromethane treatment of toxoid D all caused protein unfolding.

The results of toxoid E suggest that hydrogen peroxide modified the Trp residues, and thus altered their fluorescent properties. In addition, it is also credible that some specific Tyr and/or Trp modifications contributed to the marked reduction in fluorescence intensity of the glutaraldehyde and tetranitromethane treated toxoids, C and D.

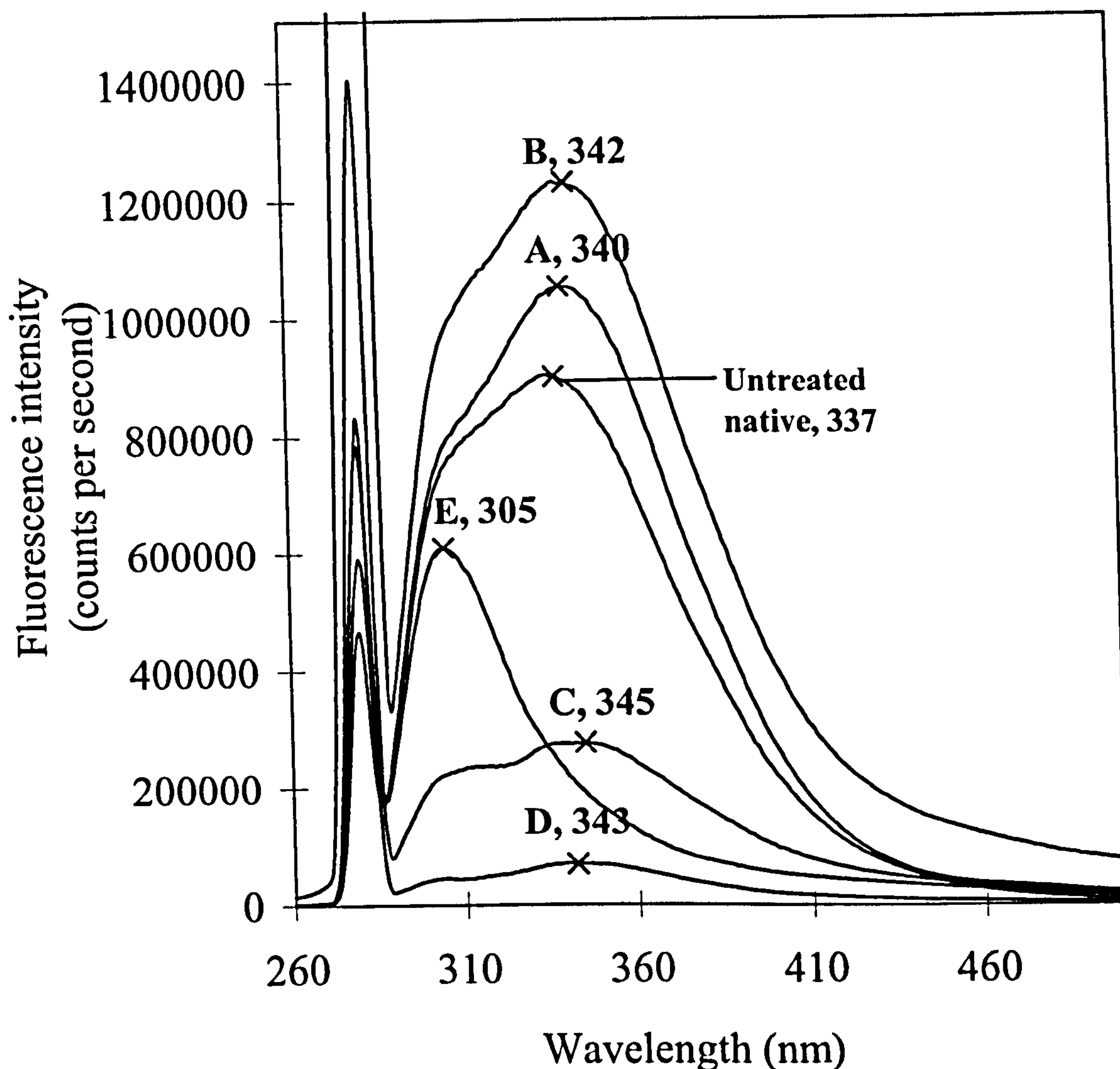


Figure 4.2 Effect of different different toxoiding procedures on PT fluorescence Pertussis toxin (PT) toxoids (30 µg/mL) obtained from five different manufacturers (A E), and untreated native PT were analysed by fluorescence spectroscopy in PBS, pH 7.5 with excitation at 280 nm. Fluorescence was scanned from 260 - 500 nm, and spectra corrected by subtraction of the PBS base-line spectra. Each manufacturer employed a different PT toxoiding agent: A - genetically detoxified plus low formaldehyde treatment, B - glutaraldehyde plus formaldehyde treatment, C - glutaraldehyde treatment, D - tetranitromethane treatment, E - hydrogen peroxide treatment. The different toxoids are labelled by letter along with their corresponding fluorescence maximum values (nm) which are marked with a cross on each spectra.

4.2.2 The effect of different chemical toxoiding on PT subunit cross-linking and epitope integrity

Analysis of PT toxoids by SDS-PAGE and Western blotting

Three different toxoids, B, C and E, and an untreated native PT sample (each of ~ 5 µg) were subject to SDS-PAGE in tris-glycine SDS running buffer using a 14 % tris-glycine acrylamide gel, under non-reducing conditions. The gels were blotted onto nitrocellulose membranes and each screened with a mAb, specific for an epitope on the PT subunits S1 – S5, or stained with Coomassie blue. Molecular weights were determined relative to protein molecular weight standards that were run on each gel.

The Coomassie-stained gel illustrates the typical four-band pattern of untreated native PT (Figure 4.3), with distinct bands of relative molecular weights 26.5 kDa (S1), 24.1 (S2), 22.2 (S3) and 13.4 (S4 and S5). The aldehyde-treated toxoids B and C, produced a smear of protein from close to the gel limit (250 kDa) to ~ 20 kDa, and protein bands corresponding to the molecular weight of subunits S4/5, S1 and higher. Toxoid B produced denser higher molecular weight bands than toxoid C. The hydrogen peroxide-treated toxoid E did not produce any high molecular weight bands but produced a very faint band at ~ 32 kDa and slightly darker bands at 29 kDa, 24 kDa and 14 kDa.

In direct correlation with the SEC/UV results, the results from SDS-PAGE suggested that aldehyde treatment of toxoids B and C promoted the production of heterogeneous high molecular weight aggregates, and in addition revealed that the high molecular weight

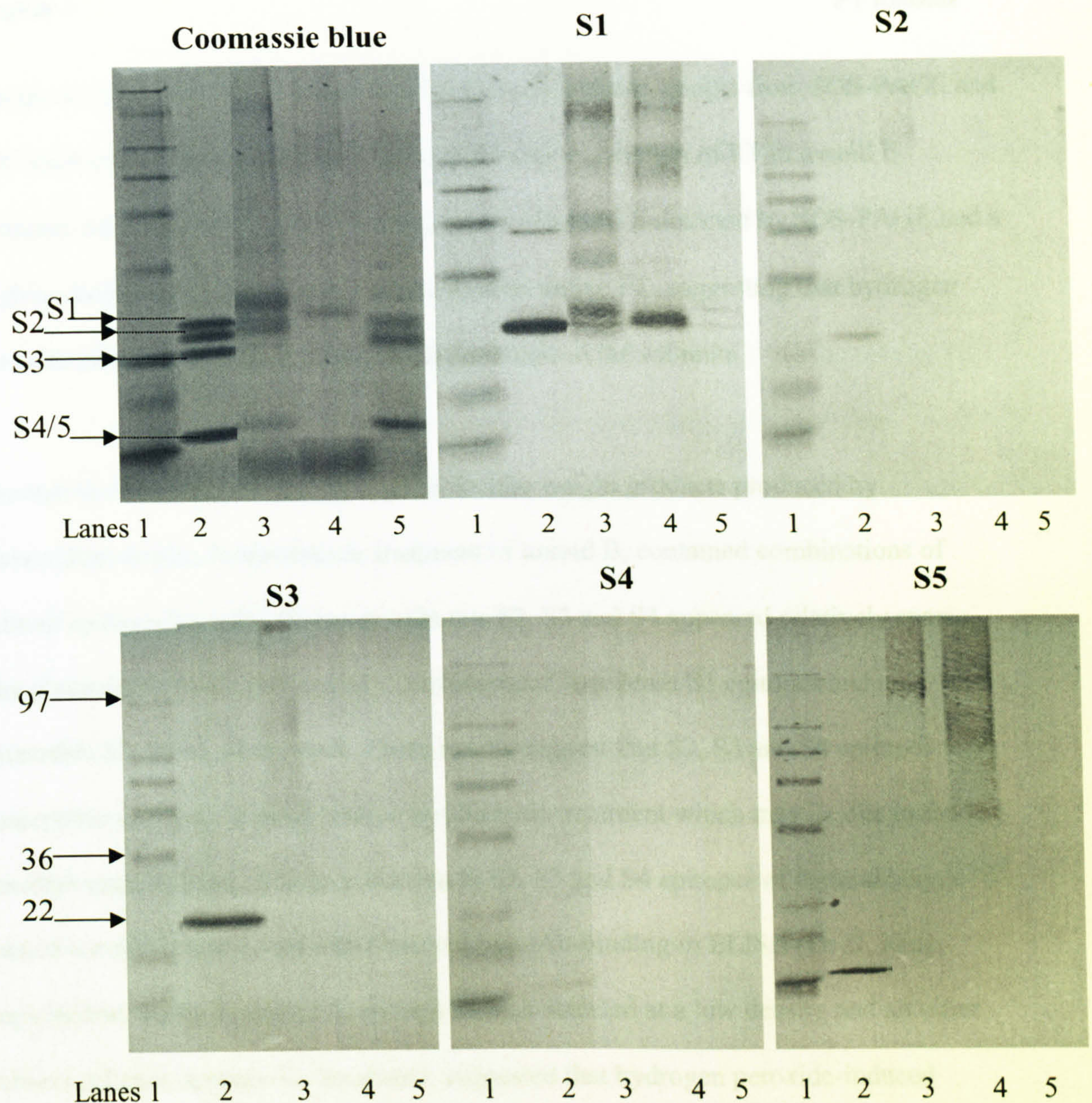


Figure 4.3 The effect of different chemical detoxification procedures on subunit cross-linking

Chemically detoxified PT antigen from 3 different companies, B, C and E (lanes 3, 4 and 5 respectively), an untreated native PT sample (lane 2) and molecular weight standards (lane 1; labelled in kDa) were analysed by SDS-PAGE and stained with Coomassie blue stain, or blotted on to nitrocellulose and screened using one of five Mabs specific for epitopes on subunits S1 – S5. Each manufacturer employed a different PT toxoiding agent: B - glutaraldehyde plus formaldehyde treatment, C - glutaraldehyde treatment, E - hydrogen peroxide treatment

species are associated by covalent cross-linking. In contrast, results from SDS-PAGE and SEC showed that the molecular weight and subunit association of PT in toxoid E remained relatively unchanged. The four bands of toxoid E detected by SDS-PAGE had a slightly shorter migration distance than untreated native PT, suggesting that hydrogen peroxide alters the charge, hydrophobicity or shape of the subunits.

Western blotting showed that the high molecular weight products produced by glutaraldehyde plus formaldehyde treatment of toxoid B, contained combinations of subunit epitopes S1 – S5. However, subunits S2, S3 and S4 appeared relatively sparse. The glutaraldehyde-treated toxoid C encompassed less dense S1 epitopes and no detectable S2, S3 or S4 epitopes. These results suggest that S2, S3 and S4 epitopes were susceptible to chemical modification by aldehyde treatment which may be due to the covalent cross-linking. A large reduction in S2, S3 and S4 epitopes of these aldehyde-treated toxoids B and C was also observed by mAb-binding in ELISA (Dr D. Xing, unpublished work). In toxoid E, epitope S1 was detected at a low density and all other subunit epitopes appeared to be absent, suggested that hydrogen peroxide-induced modifications are detrimental to the integrity of PT epitopes. In this toxoid E, a decrease in all subunit epitopes, S1 – S5, was also observed by mAb binding in ELISA (Dr D. Xing, unpublished work).

4.2.3 The effect of different chemical toxoiding on murine anti-PT IgG antibody response and protective efficacy

Analysis of anti-PT IgG antibody response by ELISA

Sample groups of five mice (strain, NIH) were each immunised with one immunisation dose (5 µg) of the five different toxoids, A – E. After five weeks, the mice were terminally bled and serum samples prepared. The PT-specific total IgG, and IgG1 and IgG2a subclass antibodies in the serum samples were quantified using ELISA.

The genetically inactivated FA-treated toxoid A induced the highest IgG response (Figure 4.4), followed by a two-fold lower response induced by the glutaraldehyde plus formaldehyde-treated toxoid B. The glutaraldehyde- and tetranitromethane-treated toxoids, C and D, induced similar total IgG responses, which were six-fold lower than for toxoid A. Finally, the hydrogen peroxide-treated toxoid E induced the lowest IgG response, 13-fold lower than for toxoid A.

The decrease in total IgG response of toxoid C, compared with toxoid B, correlates with its diminished epitope density, as illustrated by results of Western blotting. In particular, toxoid C possessed less of the S1 epitope, which is known to be a highly protective epitope of PT (Sato, Sato, and Ohishi, 1991; Sato and Sato, 1990). The low IgG response induced by hydrogen peroxide-treated toxoid E may also be a consequence of its exceptionally low density of subunit epitopes, as detected by Western blotting.

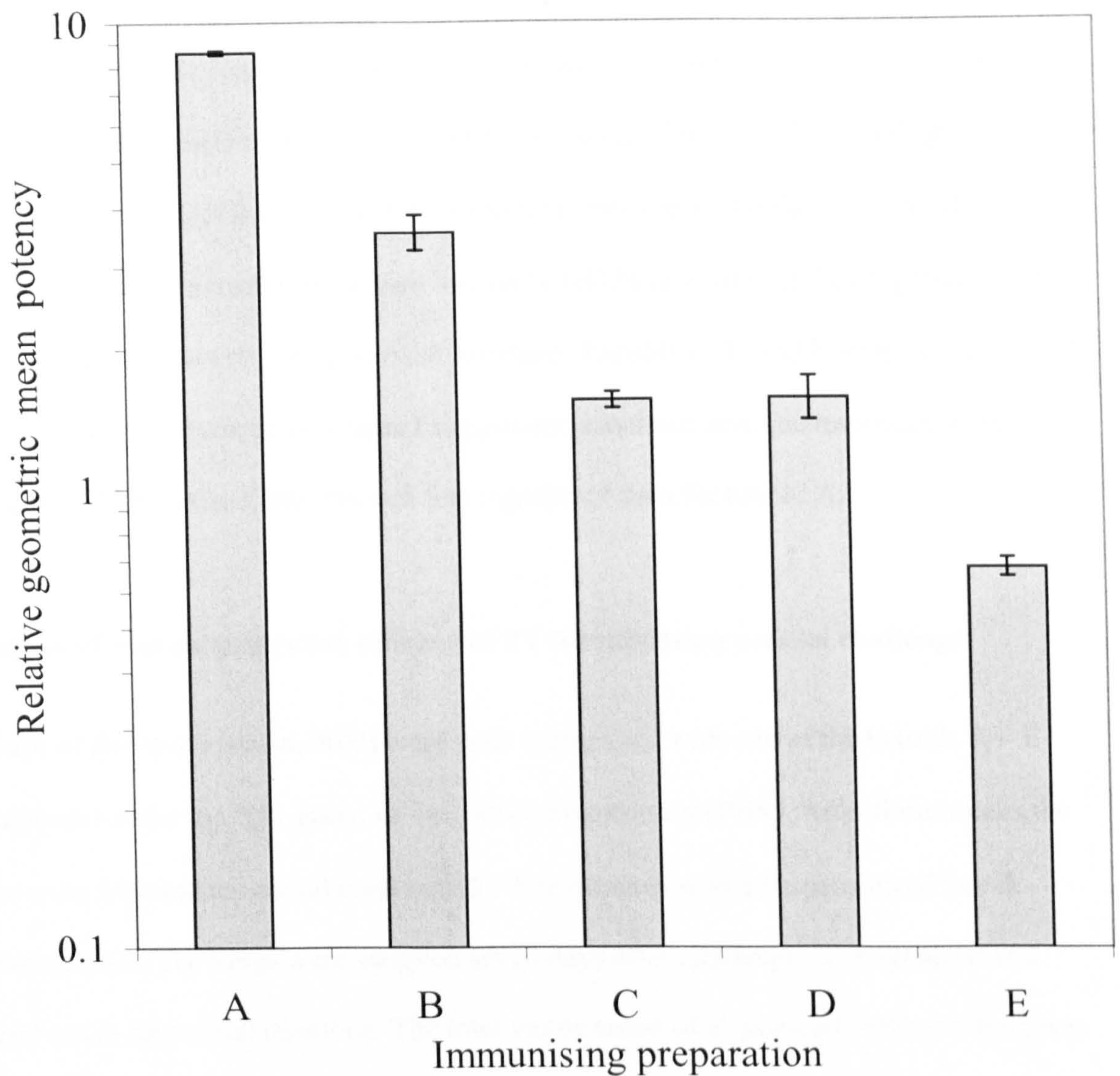


Figure 4.4 The effect of different PT toxoiding procedures on total IgG production

Serum was collected from groups of five mice, each group immunised with 5 μ g of a different pertussis toxin (PT) toxoid obtained from five different manufacturers (A - E). PT-specific total IgG was quantitated by ELISA relative to a PT reference serum, and expressed as the relative geometric mean of the five serum samples with one geometric standard deviation, indicated by error bars. Each manufacturer employed a different PT toxoiding agent: A - genetically detoxified plus low formaldehyde treatment, B - glutaraldehyde plus formaldehyde treatment, C - glutaraldehyde treatment, D - tetranitromethane treatment, E - hydrogen peroxide treatment.

The IgG1 and IgG2a responses to the toxoids varied, and did not necessarily follow the trend of the total IgG (Figure 4.5). Toxoid A gave a high IgG1/IgG2a ratio (Figure 4.6) which is an indication that the immune response it induced was polarised towards a Th2 type of response. Toxoid B had a very low IgG1/IgG2a ratio (0.4) indicating that it may have induced a relatively Th1 polarised response. Toxoids C, D and E all gave an IgG1/IgG2a ratio much greater than 1 suggesting that these immune responses were also relatively Th2 polarised, but this was less significant than for toxoid A.

Analysis of murine protective efficacy of PT toxoids using aerosol challenge

Groups of five mice (strain, NIH) were each immunised with one of the toxoids A – E (5 µg/dose) as for the IgG assay, or with a negative control (PBS). After three weeks the mice were exposed to aerosol challenge for five minutes with a suspension of live *B. pertussis* cells. The lungs were sampled seven days after challenge, homogenized and plated out in four serial dilutions. The total viable count of *B. pertussis* cells per lung was determined and the geometric mean count per lung, and geometric standard deviation, for each sample group of five mice was calculated.

The protective efficacy of the five different toxoids was in the order A, B, C, D and E (Figure 4.7). The genetic toxoid A treated with low FA was by far the most protective, and in this group of mice the bacterial count was 50-fold less than that detected in the negative control group. Protection provided by glutaraldehyde plus FA-treated toxoid B was four-fold lower than for toxoid A, and glutaraldehyde-treated toxoid C was ten-fold

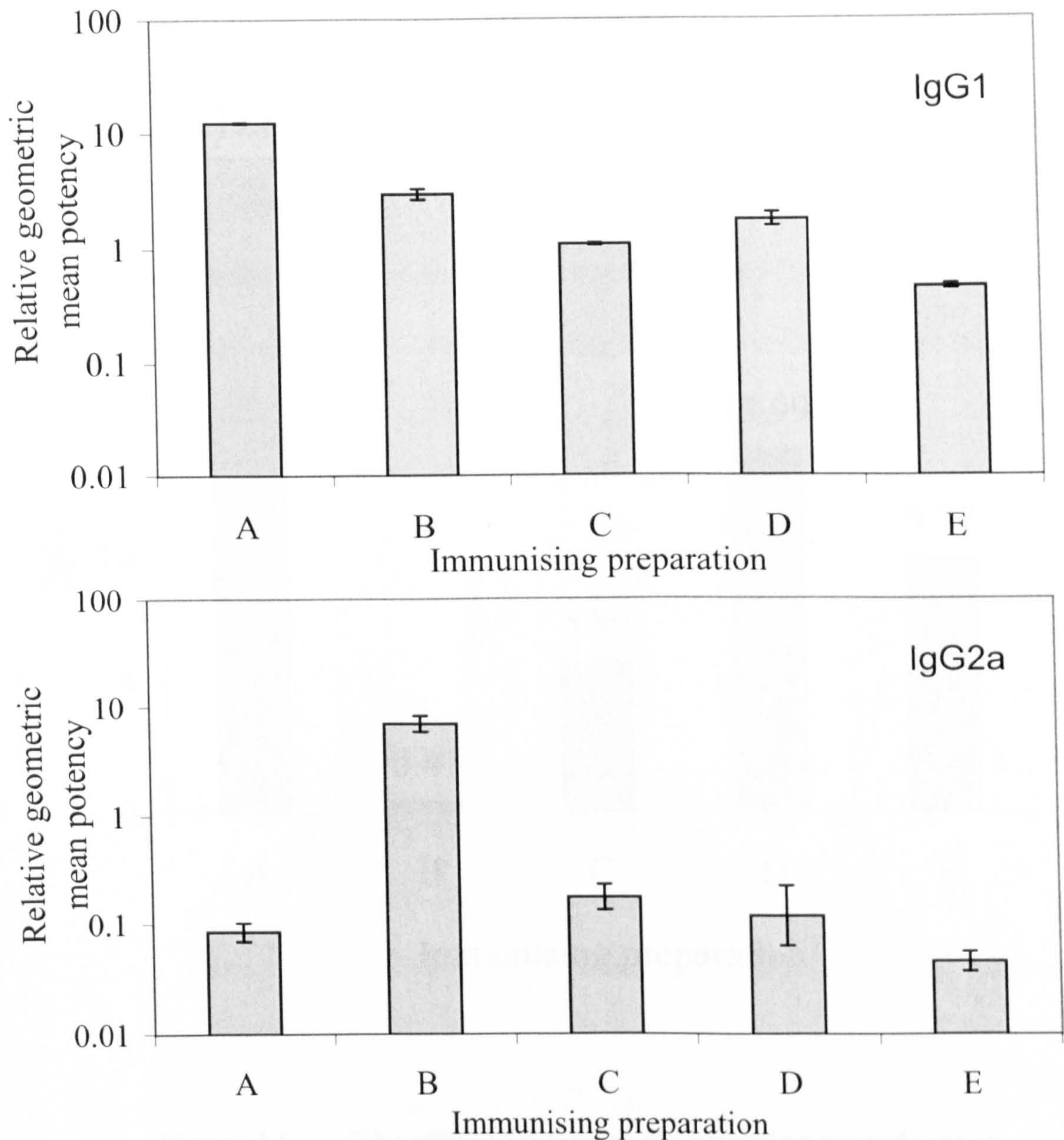


Figure 4.5 The effect of different PT toxoiding procedures on IgG1 and IgG2a production

Serum was collected from groups of five mice, each group immunised with 5 μ g of a different pertussis toxin (PT) toxoid obtained from five different manufacturers (A - E). PT-specific IgG1 and IgG2a subclasses were quantitated by ELISA relative to a PT reference serum, and expressed as the relative geometric mean of the five serum samples with one geometric standard deviation, indicated by error bars. Each manufacturer employed a different PT toxoiding agent: A - genetically detoxified plus low formaldehyde treatment, B - glutaraldehyde plus formaldehyde treatment, C - glutaraldehyde treatment, D - tetranitromethane treatment, E - hydrogen peroxide treatment.

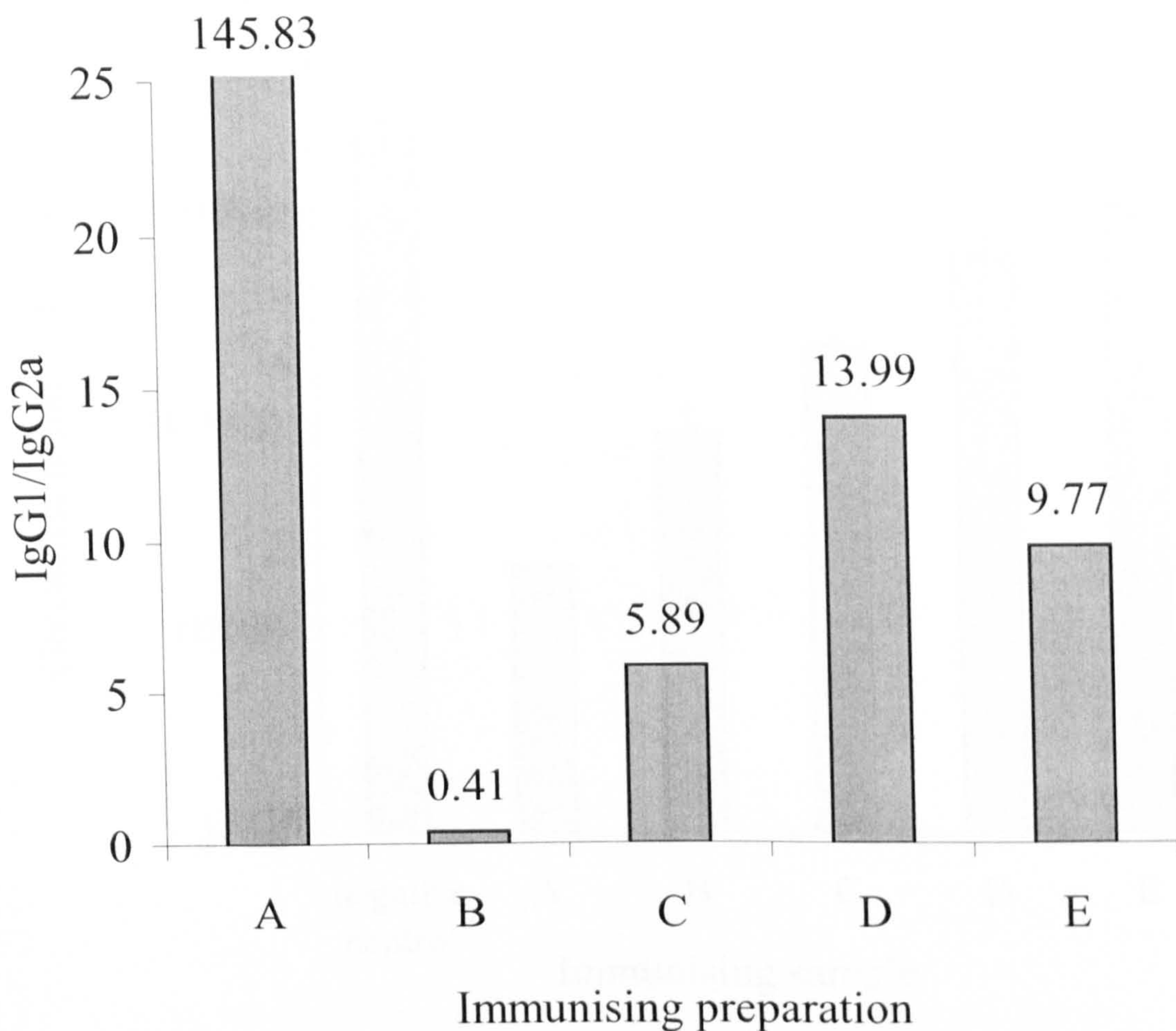


Figure 4.6 The effect of different PT toxoiding procedures on on the IgG1/IgG2a ratio

Five mice were each immunised with 5 µg of one of five different company's PT antigen (A - E). PT-specific IgG1 and IgG2a in the mice sera were quantitated by ELISA, relative to a PT reference, and the geometric mean potency of the five serum samples per group determined. The geometric means are expressed here as IgG1/IgG2a ratio. Each manufacturer employed a different PT toxoiding agent: A - genetically detoxified plus low formaldehyde treatment, B - glutaraldehyde plus formaldehyde treatment, C - glutaraldehyde treatment, D - tetranitromethane treatment, E - hydrogen peroxide treatment.

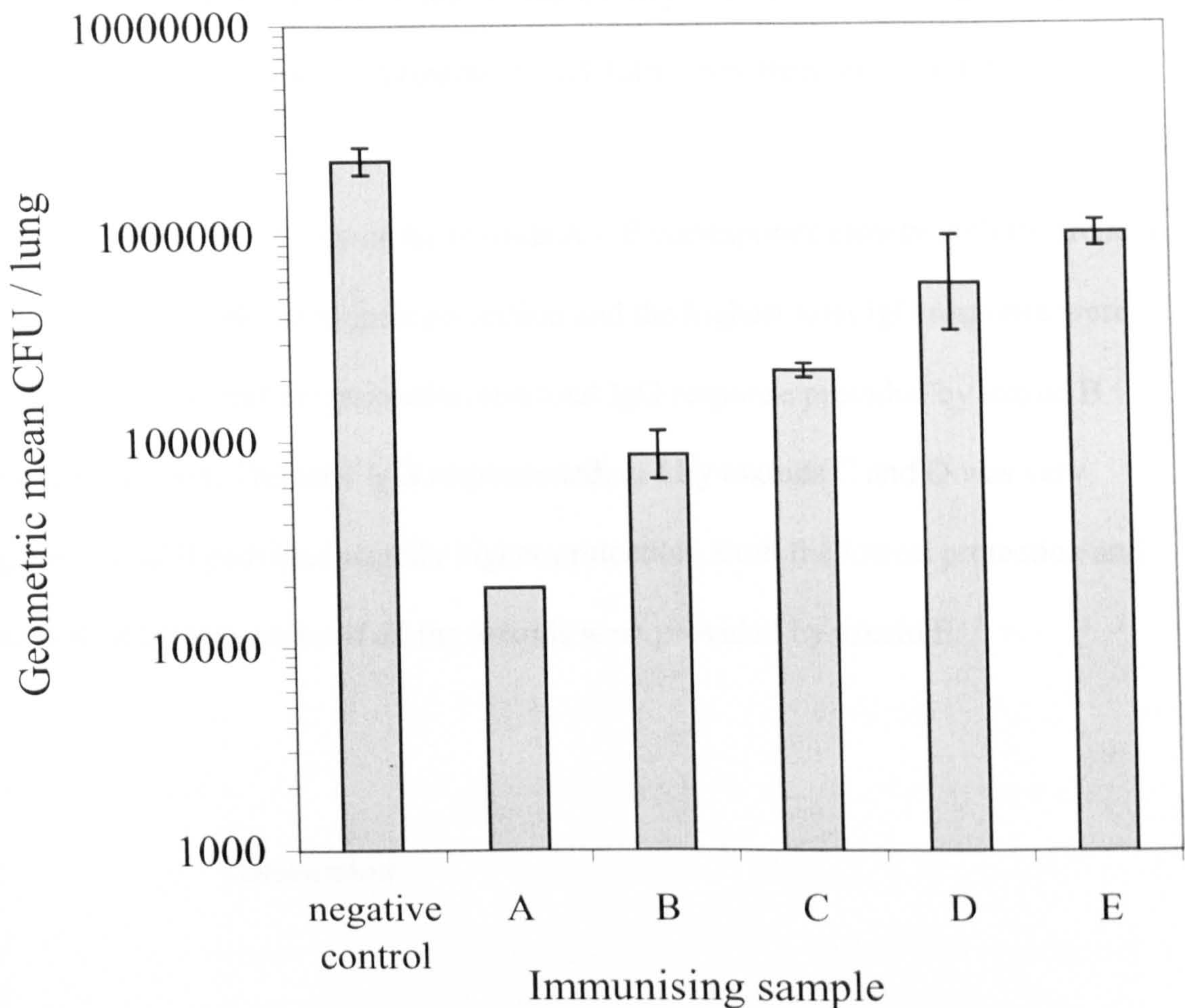


Figure 4.7 Protective efficacy of PT toxoids

Groups of five NIH mice were aerosol challenged with live *B. pertussis* cells three weeks after immunisation with pertussis toxin (PT) toxoids, A - E (from five different manufacturers). Their lungs were sampled seven days after challenge, the number of colony forming units (CFU) per lung were determined and the geometric mean CFU and geometric standard deviation (expressed as error bars) for each group of five mice were calculated. 100 % infection is equivalent to 2.2×10^6 CFU/lung. Each manufacturer employed a different PT toxoiding agent: A - genetically detoxified plus low formaldehyde treatment, B - glutaraldehyde plus formaldehyde treatment, C - glutaraldehyde treatment, D - tetranitromethane treatment, E - hydrogen peroxide treatment.

This experiment was carried out by Cathy Canthaboo, NIBSC, 1997.

lower. Tetranitromethane- treated toxoid D and hydrogen peroxide-treated toxoid E provided a relatively low level of protection ~ 25-fold lower than for toxoid A.

The trend in protective efficacy of the toxoids A – E corresponds closely with the trend in total IgG response. Both the highest protection and the highest total IgG response were induced by toxoid A, and the protection and total IgG response provided by toxoid B were the next highest. The total IgG response induced by toxoids C and D was very similar but toxoid D provided slightly higher protection. Both the lowest protection and the lowest total IgG response of all the toxoids were provided by toxoid E.

4.3 Discussion

In order to address the role of toxoids in modulating the immune response, five commercial toxoids were analysed by structural and immunological techniques.

Genetically detoxified PT with 0.035 % FA (toxoid A)

Toxoid A encompassed heterogeneous PT species of slightly higher molecular weight than untreated native PT as illustrated in the SEC results. Since untreated mutant PT has been shown to be structurally similar to untreated native PT (Nencioni *et al.*, 1990), it follows that it was the FA treatment which caused modification of the mutant PT toxoid. There appeared to be some unfolding of the toxoid species as determined by fluorescence. Although toxoid A was not analysed by Western blotting in this study,

mAb-binding was previously analysed by ELISA (Dr D. Xing, unpublished work) and showed that there was a high density of all subunit epitopes as compared with untreated native PT. In addition, toxoid A induced the highest total IgG response of all the toxoids tested, and the highest level of protection as determined by aerosol challenge.

Glutaraldehyde plus formaldehyde-treated PT (toxoid B)

Toxoid B was composed of species much more heterogeneous, and of considerably higher molecular weight than untreated native PT, as determined by SEC. Fluorescence spectroscopy also showed that at least a proportion of the toxoid species was unfolded compared to the untreated mutant PT. SDS-PAGE and Western blotting results of toxoid B confirmed the presence of high molecular weight species, and demonstrated that they were associated by inter-subunit covalent cross-linking. In addition, these results revealed that some subunit epitopes, in particular the protective epitope on S1, were maintained in even the very highest molecular weight species. The presence of immune epitopes in toxoid B may account for its high immunogenicity, as illustrated by the relatively high IgG response and the high protection against aerosol infection, which was second only to toxoid A.

Glutaraldehyde-treated PT (toxoid C)

Toxoid C contained very high molecular weight material even compared to toxoid B, as told by the SEC spectra. As with toxoids A and B, the red-shifted intrinsic fluorescence implied that these high molecular weight species were composed of unfolded protein. In addition, intrinsic fluorescence intensity was unusually low, also suggesting that the

aromatic amino acids had been modified and their fluorescence properties were altered. Western blotting demonstrated that the high molecular weight species formed by glutaraldehyde treatment were associated by inter-subunit covalent cross-linking. However, toxoid C encompassed a lower density of epitopes compared with toxoid B. This correlates with the lower IgG response and decreased protectivity of toxoid C, compared with that of toxoid B.

The variation of PT cross-linking, aggregation and epitope modification between the aldehyde-treated toxoids, A, B and C, may depend on differences in the detoxification protocol such as the concentration of aldehydes employed, the presence of amino acid additives, and the temperature and time of incubation. For example, toxoids B and C contained higher molecular weight material than toxoid A, which is very likely to be a consequence of the higher concentrations of aldehyde treatment required for detoxification of native PT. This proposal is in agreement with experiments which suggested that aldehydes such as formaldehyde and glutaraldehyde can cause an increase in the molecular weight of *B. pertussis* protein antigens, including PT and FHA (Bolgiano *et al.*, 1999). In addition, FA-treatment has been shown to generate covalent cross-links in bovine serum albumin (Tome, Kozlowski, and Mabon, 1985). Also, in the present study, the more severely aldehyde-treated toxoids, B and C, had a lower immunogenicity and protective efficacy than toxoid A. In correlation with this, a previous study of mutant PT showed that increasing FA-treatment caused a decrease in its S1 protective epitope recognised by mAb 1B7, and a reduction in its ability to induce neutralising antibodies *in vivo* (Nencioni *et al.*, 1991).

Tetranitromethane-treated PT (toxoid D)

Toxoid D was composed of a large proportion of high molecular weight material as observed by SEC. In agreement with these results, tetranitromethane has previously been reported to cause aggregation in a variety of proteins including rabbit gamma-globulin and collagen (Doyle, Bello, and Roholt, 1968). As for the aldehyde-treated toxoids, the intrinsic fluorescence of toxoid D also suggested that it was composed of at least a proportion of unfolded protein. An additional possibility for the modified fluorescence spectra may be the chemical modification of Trp and Tyr residues by tetranitromethane, which may alter their fluorescence properties. This is very likely since tetranitromethane has been shown to react with both Trp and Tyr residues in proteins (Muhlrad, Corsi, and Granata, 1968; Riordan, Sokolovsky, and Vallee, 1967; Cuatrecasas, Fuchs, and Anfinsen, 1968). Previous analysis of this toxoid by mAb in ELISA showed a significant loss in the S1 epitope and some decrease in the S3 and S4 epitopes (Dr D. Xing, unpublished work). These modifications of PT correspond to the reduction in immunogenicity and protective efficacy of toxoid D.

Hydrogen peroxide-treated PT (toxoid E)

Hydrogen peroxide treated PT was shown by SEC to be comprised of protein of similar molecular weight to untreated native PT. No additional peaks were observed, suggesting that the molecular association between the subunits had not been significantly altered. It is likely that slightly later elution time in comparison with untreated native PT is due to a difference in the molecular shape or charge of PT as a result of modification with

hydrogen peroxide. Toxoid E displayed a peculiar intrinsic fluorescence, compared with the other toxoids, showing no signs of Trp fluorescence, which suggested that the Trp residues of PT were chemically modified. These findings directly correlate with the known activities of hydrogen peroxide, which is a powerful oxidative agent of many organic compounds, and in proteins can attack cysteine, cystine, methionine, Trp and Tyr (Means.G.E. and Feeney.R.E., 1971b). It has also been previously shown to degrade Trp in Trp-containing peptides and in lysozyme (Simat and Steinhart, 1998). It is likely that the oxidative effects of hydrogen peroxide are detrimental to the integrity of PT epitopes, since only a low density of S1 subunit epitopes, out of a total of five subunit epitopes screened, were detected by Western blotting. A previous study has also identified hydrogen peroxide-induced destruction of neutralising epitopes on the S1 subunit and B-oligomer of the PT molecule (Ibsen, 1996). In correlation with these results the total IgG response induced by toxoid E was very low, 14-fold lower than that induced by toxoid A, and it gave the poorest protection against aerosol challenge.

Correlates of immune response to combined vaccines

Our results demonstrated that the antibody response to the different toxoids, starting with the highest, was in the order: mutant PT stabilised with FA, glutaraldehyde plus FA-treated PT, glutaraldehyde-treated PT/tetranitromethane-treated PT, hydrogen peroxide-treated PT. These results agree with those from a previous study of combined DTP vaccines containing acellular pertussis components (Edwards *et al.*, 1995). The study showed that combined DTP vaccines induced antibody responses, starting with the

highest, in the order (referenced by PT toxoid): mutant PT stabilised with FA, glutaraldehyde-treated PT, FA-treated PT, tetranitromethane-treated PT.

The results of this chapter illustrate that chemical toxoiding agents produce a variety of structural modifications of PT. Chemical detoxification is likely to be the result of a combination of specific chemical reactions causing local damage to functional groups, and more widespread non-specific cross-linking and aggregation which possibly masks or conformationally alters functional groups of PT. The detoxifying modifications of PT also affect the epitopes of the toxoid, the extent and type of immune response they induce and ultimately their ability to protect against infection. These results give proof that modifications of PT molecules are influenced by both the chemical toxoiding agent and the toxoiding method, and this is likely to affect the overall efficacy of acellular pertussis vaccines. This study prompted more in-depth investigations into the effects of formaldehyde on the structure and immunogenicity of PT (described in Chapters 5 and 6).

5.1 Introduction

Pertussis toxin (PT) is a major virulence factor of the whooping cough bacterium *B. pertussis* (Monack *et al.*, 1989). It belongs to the AB class of ADP-ribosylating protein toxins, which are characterised by their distinct functional binding and catalytic domains. The PT holotoxin, of molecular mass 105 kDa (Stein *et al.*, 1994a) comprises a catalytic A (S1)-subunit (26 kDa), which ADP-ribosylates certain G-proteins, disrupting cell-receptor signalling, and a pentameric B-oligomer (79 kDa), for binding to the tracheal epithelium. The B-oligomer is composed of five subunits, S2, S3, two copies of S4 and S5, which have molecular weights of 22, 22, 12 and 11 kDa respectively (Stein *et al.*, 1994a). These subunits are primarily assembled as two dimers, S2-S4 and S3-S4, and the contacts forming these dimers are extensive anti-parallel beta-sheet interactions, forming the most extensive interactions of the PT molecule. The dimers are associated together with S5 mainly through antiparallel β -sheet interactions, to form a pentameric asymmetric ring structure with a central pore. The catalytic S1-subunit penetrates the pore with its carboxyl terminal tail positioning it on top of the ring structure.

PT in a chemically or genetically detoxified (PT-9K/129G) form is the primary component of all current acellular whooping cough vaccines. Formaldehyde (FA) is used

in some acellular pertussis vaccine preparations to detoxify native PT (Edwards *et al.*, 1995; Sato, Kimura, and Fukumi, 1984), and is also used in lower concentrations to stabilise the genetically detoxified PT (PT-9K/129G), herein referred to as mutant PT (Edwards *et al.*, 1995; Nencioni *et al.*, 1991). Most studies on the effects of FA on PT have been carried out on mutant PT and have largely focused on changes in haemagglutinating activity and mitogenicity (Nencioni *et al.*, 1991) as well as effects on T- and B-cell epitopes (Nencioni *et al.*, 1991; Di Tommaso *et al.*, 1994; Cropley *et al.*, 1995; Ibsen, 1996; Nencioni *et al.*, 1990).

Preliminary structural studies suggest that FA treatment of a variety of protein antigens may cause the production of high molecular weight species (Bolgiano *et al.*, 1999; Bolgiano *et al.*, 2000; Fowler *et al.*, 2000; Manetti *et al.*, 1997). The chemistry of amino acid side chain modifications arising from FA treatment has also been explored (Means.G.E. and Feeney.R.E., 1971a; Tome and Naulet, 1981; Petre *et al.*, 1996). However, there is little understanding of the effect of FA on secondary, tertiary and quaternary protein structure despite its widespread use in vaccine preparations. A thorough understanding of the molecular forms produced by FA-detoxification is a necessary prelude to determining the roles of different molecular forms in directing the immune response. In addition, guidelines by the World Health Organisation have emphasised the importance of physico-chemical testing for the standardisation of acellular pertussis vaccines (Arciniega *et al.*, 1998). The advantages of establishing molecular correlates of immunogenicity or protection also extend to the development of physico-chemical procedures as alternatives to animal experiments.

In the present study, a detailed physico-chemical characterisation of the effects of FA on PT was undertaken. Firstly, mutant and native PT were compared, before and after FA-treatment, using SEC/UV, fluorescence spectroscopy and CD. In this analysis the native and mutant PT were treated with relatively high concentrations of FA (0.25 – 1.00 % FA) using a long incubation time of 7 days at 37 °C, due to the putative toxicity of the native PT preparations. For safety reasons mutant PT preparations were used for the remainder and majority of the study, and were treated with lower FA concentrations (0.01 – 0.50 % FA), and a shorter incubation time of 48 hours, in order to characterise the effects of FA from low stabilising concentrations to higher toxoiding concentrations. The effect of FA on size and subunit association of PT was assessed using SDS-PAGE, SEC/UV, AUC and SEC/MALLS. In addition, the effects of FA on the protein conformation of PT were explored using fluorescence spectroscopy.

5.2 Results

5.2.1 Comparison of the effect of FA on native and mutant PT

SEC/UV of FA-treated native and mutant PT

Mutant and native PT preparations, untreated and treated with 0.25 and 1.00 % FA (high FA-treatment, Chapter 2, section 2.2.3) were subject to SEC/UV using an FPLC system

equipped with a Superdex 200 column, at a flow rate of 0.5 mL/min in PBS buffer, pH 7.5. The elution of protein was detected both at 214 and 280 nm, and the 214 nm trace is presented (Figure 5.1)

For both untreated mutant and native PT preparations comparable SEC spectra were obtained and the majority of protein eluted from the SEC column as a main broad peak at 18.0 mL, with a trailing shoulder (Figure 5.1). For both 0.25 % FA-treated mutant and native PT preparations, the main peak became broader and eluted significantly earlier at 14.2 mL, and an additional peak for both preparations appeared at 11.8 mL. Increasing FA treatment to 1.00 % caused the main peak of both mutant and native PT preparations to elute even earlier at 13.4 mL. The increasingly early elution of the mutant and native PT molecules suggests that FA treatment caused the PT molecules to associate together to form much larger species. The broadening of the main peak and appearance of another distinct peak illustrate the increasing heterogeneity of protein species size with increasing FA. Moreover, the mutant and native PT preparations had very similar elution patterns, indicating that they were structurally similar and were modified in the same way with FA treatment to form analogous molecular assemblies.

Fluorescence spectroscopy of native and mutant PT

Mutant and native PT preparations, untreated and treated with 1.00 % FA (high FA-treatment, Chapter 2, section 2.2.3) were subject to fluorescence spectroscopy using a single photon-counting spectrofluorimeter by λ_{ex} of 280 nm.

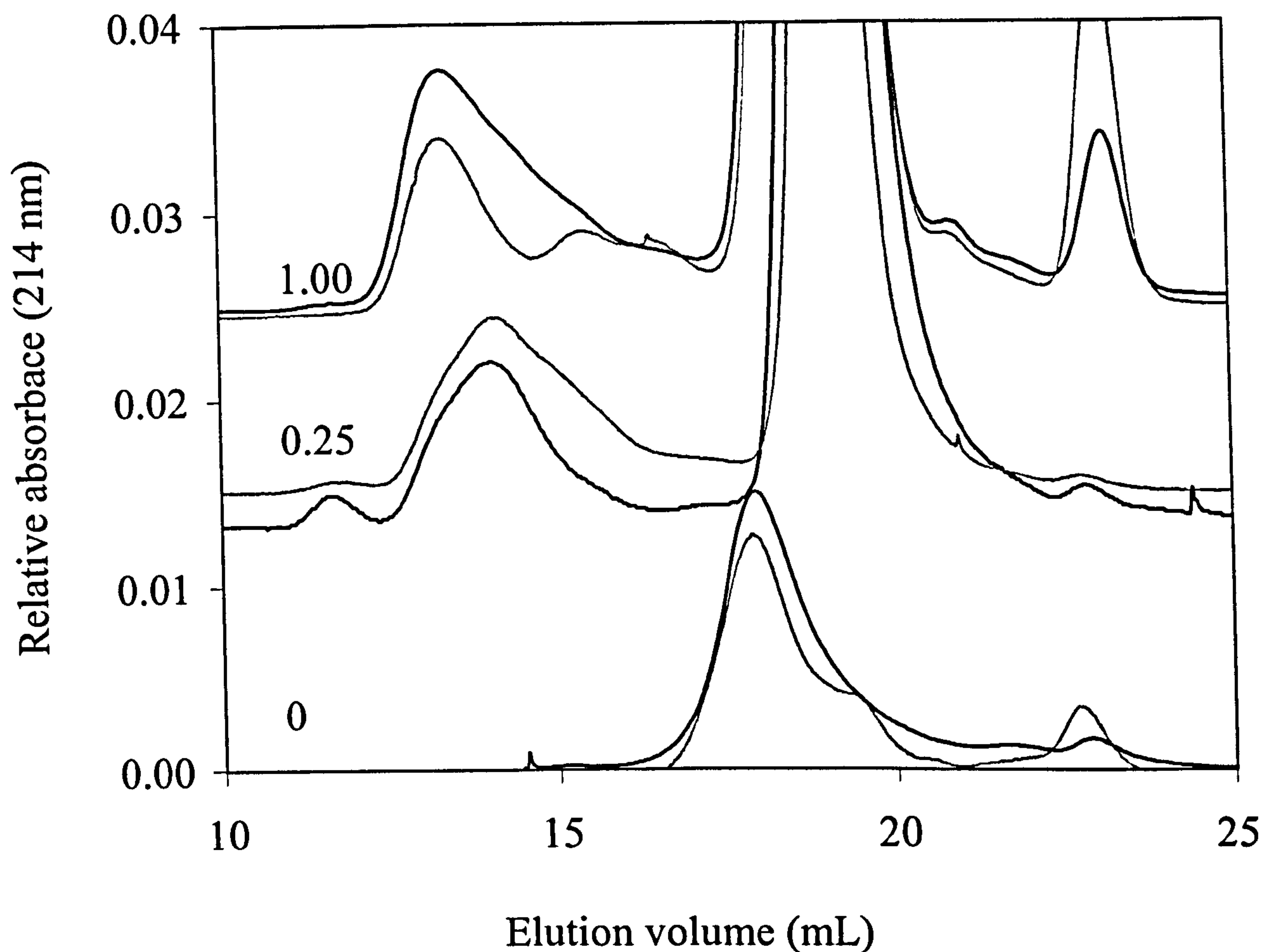


Figure 5.1 The effect of FA on the size of native and mutant PT

Samples (15 μ g protein in 200 μ L PBS, pH 7.5) of native (black trace) and mutant (grey trace) pertussis toxin (PT), untreated and treated with 0.25 and 1.0 % formaldehyde (FA) were analysed using size exclusion chromatography with UV detection (SEC/UV). The FPLC system was equipped with a Superdex 200 column and samples were eluted in PBS, pH7.5. Absorbance at 214 nm is shown.

The fluorescence was scanned from 260 to 500 nm and the PBS, pH 7.5 buffer base line fluorescence was subtracted.

Untreated mutant and native PT displayed superimposable fluorescence spectra (Figure 5.2) with a characteristic Trp fluorescence emission wavelength maximum (F_{\max}) at 337 nm and a shoulder from Tyr fluorescence at ~ 305 nm (Seabrook, Atkinson, and Irons, 1991). With FA-treatment the F_{\max} for both the untreated and mutant PT preparations shifted to longer wavelengths (red-shift), displaying an F_{\max} of 345 nm (Figure 5.2). The fluorescence red-shift observed with FA treatment of both mutant and native PT may be indicative of protein unfolding leading to solvent exposure of Trp residues, but specific chemical modification of Trp residues with altered fluorescence properties can not be ruled out. The similarity of the corresponding FA-treated mutant and native PT preparations suggests that the mutant and native PT molecules have the same conformation and that their molecular structures are altered in a similar way with FA treatment. CD was subsequently employed as a complementary tool to fluorescence.

CD of native and mutant PT

Both native (750 $\mu\text{g/mL}$) and mutant (382 $\mu\text{g/mL}$) PT preparations were analysed by near (250 – 320 nm) and far (180 – 260 nm) UV CD, in 2 x 1cm and 1 x 0.01 cm cuvettes, for respective wavelength ranges, using a Jasco J-720 spectropolarimeter. The spectra were each corrected for dialysate buffer blank (PBS, pH 7.5) and molarity, and are expressed as molar circular-dichroic absorption ($\Delta \epsilon$).

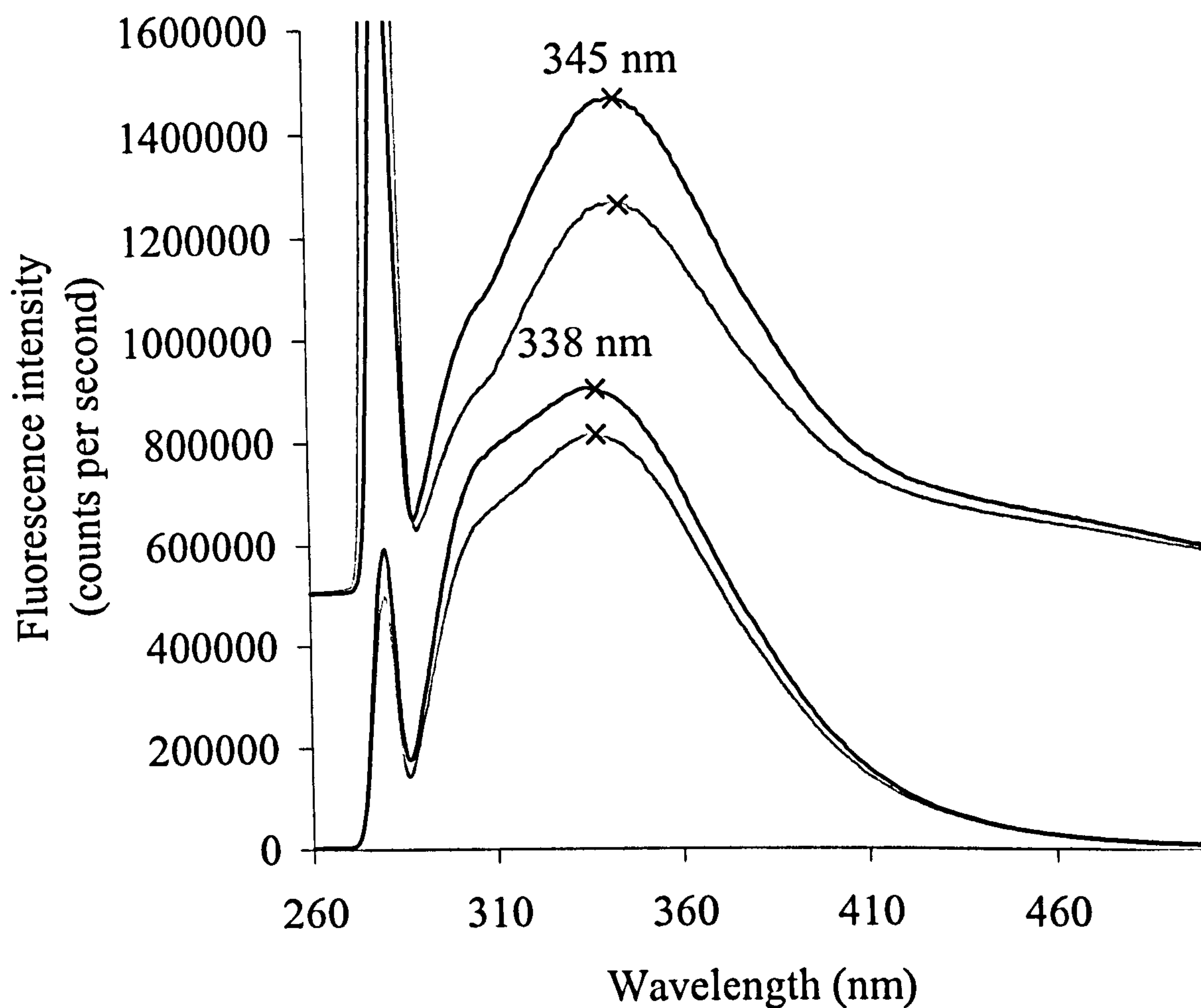


Figure 5.2 The effect of FA on the conformation of native and mutant PT. Native (black trace) and mutant (grey trace) pertussis toxin (PT) preparations (10 - 30 $\mu\text{g/mL}$) in PBS, pH 7.5, untreated or treated with 1.0 % (w/v) formaldehyde (FA), were excited at 280 nm and their fluorescence recorded from 260 - 500 nm. Spectra were corrected by subtraction of the PBS base-line spectra. Lower traces - untreated preparations; upper traces - 1.0 % FA-treated preparations

Circular dichroism is a chiroptical method, which measures the difference in absorbance of left and right circularly polarised light. In the near UV, CD bands are dominated by absorption from the aromatic side chains and disulphide linkages. CD in this range can be a sensitive indicator of changes in tertiary structure. Mutant and native PT display very similar near UV CD spectra (Figure 5.3), possessing a broad band presumably caused by the 13 di-sulphide bonds of PT. There is also particularly negative dichroic absorption caused by the large numbers of tyrosine residues present. Trp features at around 283 - 285 nm (Strickland, 1974) but this $\Delta \epsilon$ is unusually negative for proteins containing Trp. The large negative dichroic absorption may be due to the high tyrosine content of PT.

Far UV CD can be used to interpret the extent and type of secondary protein structure. Both mutant and native PT display very similar far UV CD spectra (Figure 5.3). These are typical of previous spectra obtained for PT, having a minimum at about 208 nm (Seabrook, Atkinson, and Irons, 1991; Bloemendal and Johnson, Jr., 1995), and are characteristic of $\alpha+\beta$ protein. The same native PT sample analysed in a chloride-free buffer gave a comparable far UV CD spectrum (not shown), which included data down to 180 nm. Analysis of this spectrum using the Varslc programme (variable selection method of protein secondary structure estimation) (Manavalan and Johnson, Jr., 1987) determined a secondary structure composition of 15 % α -helix and 39 % β -sheet. This is compatible with that determined from the crystal structure of PT (Stein *et al.*, 1994a), of 16 % α -helix and 37 % β -sheet (Appendix 1).

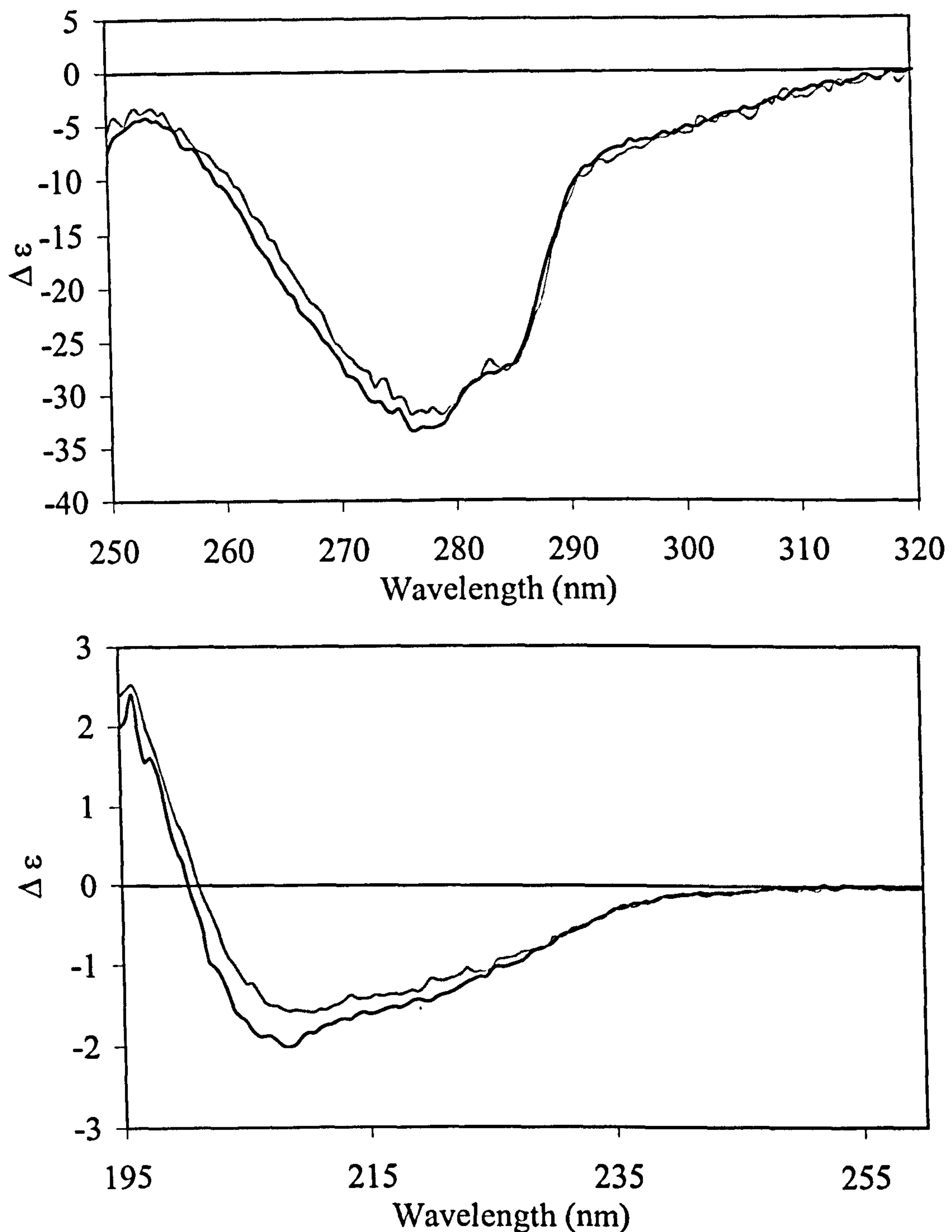


Figure 5.3 CD of untreated native and mutant PT

Circular dichroism (CD) was performed on native (750 $\mu\text{g/mL}$; black trace) and mutant (382 $\mu\text{g/mL}$; grey trace) pertussis toxin (PT) by measuring near (250 – 320 nm) and far (195 – 260 nm) UV CD, in 2 x 1 cm and 1 x 0.01 cm cuvettes, for respective wavelength ranges, using a Jasco J-720 spectropolarimeter. The spectra were each corrected for dialysate buffer blank (PBS, pH 7.5) and molarity, and are expressed as molar circular-dichroic absorption ($\Delta \epsilon$).

Overall the SEC, fluorescence spectroscopy and CD studies comparing mutant and native PT demonstrate that the untreated molecules are of similar molecular structure and quality. Furthermore the SEC and fluorescence spectroscopy results demonstrate that both mutant and native PT molecules undergo similar molecular association and conformational changes with FA treatment. To more thoroughly assess the effect of FA on PT, mutant PT preparations treated with stabilising concentrations through to toxoiding concentrations of FA (0.01 – 0.50 % FA) were analysed.

5.2.2 Effect of FA on the size and subunit association of PT

Mutant PT untreated and treated with 0.01 – 0.50 % FA (*Low FA-treatment*, Chapter 2, section 2.2.3) were analysed by four different sizing methods: SDS-PAGE, SEC/UV, SEC/MALLS and AUC.

SDS-PAGE of FA-treated PT

Mutant PT, untreated and treated with 0.035, 0.1 and 0.5 % FA (each of ~ 5 µg), were subjected to SDS-PAGE in tris-glycine SDS running buffer using a 10 well, 14 % tris-glycine acrylamide gel, under non-reducing and reducing conditions. Molecular weights were determined relative to protein molecular weight standards that were run on each gel.

Under non-reducing conditions, untreated mutant PT produced the typical 4-subunit band-pattern (Nencioni *et al.*, 1990) with relative molecular masses (M_r) of 25.6 kDa

(S1), 22.8 kDa (S2), 20.3 kDa (S3), and 9.6 kDa (S4 and S5) (Figure 5.4). In addition to this pattern, mutant PT treated with increasing FA concentrations yielded a series of defined bands of progressively higher M_r with smearing between them. Above 0.035 % (w/v) FA treatment species as large as 200 kDa were observed, which is the resolving limit of the gel. All of these features were also observed in the reducing gel (Figure 5.4).

Together the results provide evidence that FA treatment of mutant PT induces the formation of large species, some of which exceed holotoxin molecular weight. Furthermore, they reveal that the molecules are associated by stable covalent cross-linking, which is independent of disulphide bonding. The defined bands of molecular weights higher than those of monomeric subunits are indicative of homogeneous species likely to be formed by favoured covalent cross-linking between neighbouring subunits in the native structure. The smearing effect represents many species of heterogeneous size, which may encompass an array of protein species that are randomly cross-linked and/or have various side-chain modifications including decoration to different extents by lysine from the reaction mixture. It is known that in reaction with FA, a variety of amino acids, including lysine used as the FA quencher during treatment, are capable of cross-linking via their side-chain groups (Tome and Naulet, 1981).

In order to characterise any larger species that exceed the limit of the SDS-PAGE gel, or that are not irreversibly associated, the mutant PT preparations were subsequently analysed by SEC/UV.

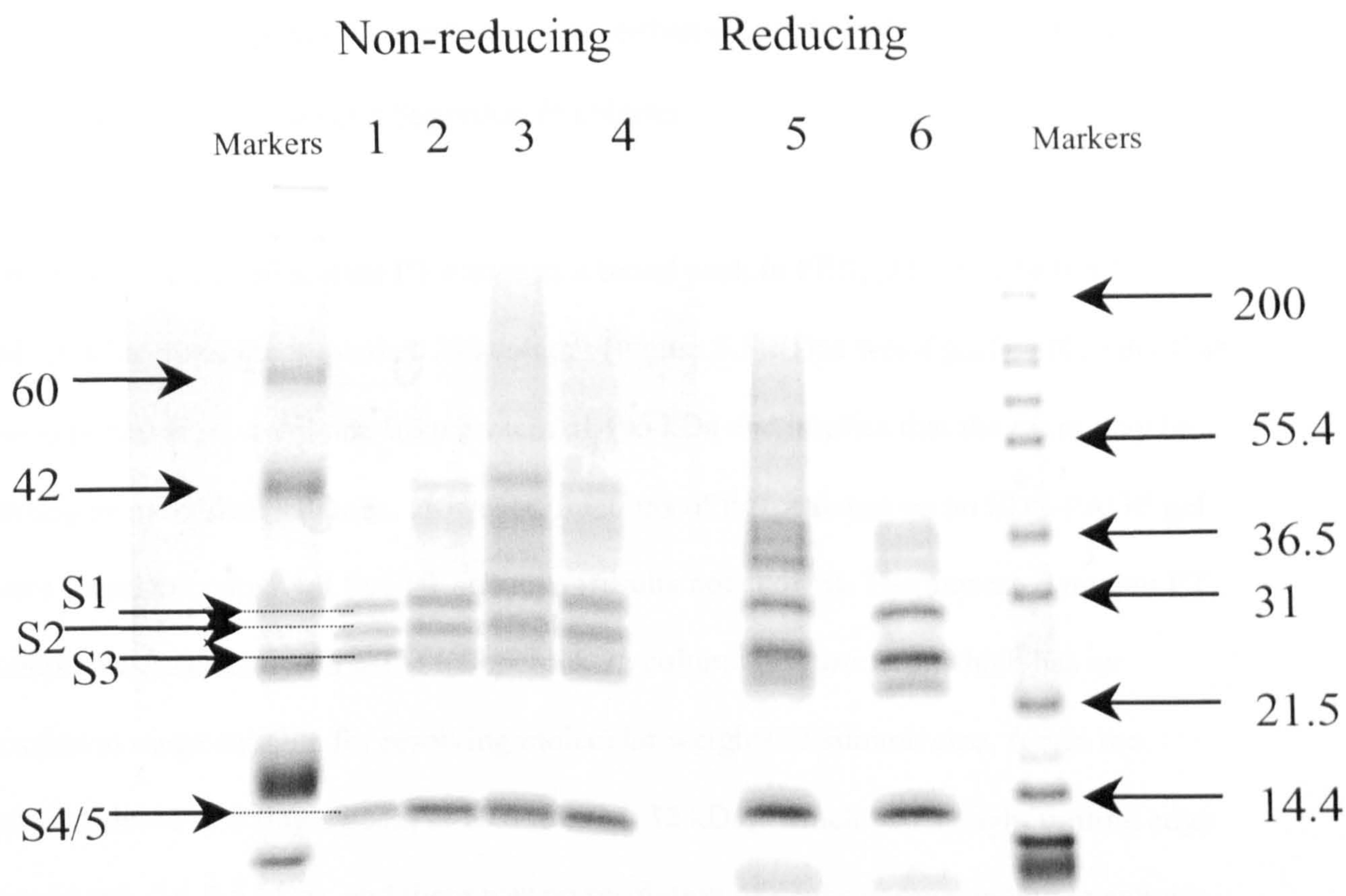


Figure 5.4 SDS-PAGE of mutant PT treated with different concentrations of FA

Mutant pertussis toxin (PT) samples (5 μ g) electrophoresed in non-reducing sample buffer were treated with 0 (lane 1), 0.035 (lane 2), 0.1 (lane 3) and 0.5 (lane 4) % (w/v) formaldehyde (FA). Two of the PT samples were electrophoresed on a second gel in reducing sample buffer: 0.5 (lane 5) and 0.035 (lane 6) % (w/v) FA. The gels were stained with Coomassie blue. Molecular weight standards ('Markers', kDa) were run in non-reducing conditions.

SEC/UV of FA-treated PT

Mutant PT untreated and treated with 0.035, 0.10 and 0.50 % FA were subject to SEC/UV using a Superdex 200 column as described above. In addition untreated mutant PT was also analysed using a Superdex 75 column.

The bulk of untreated mutant PT eluted as a broad peak in PBS, pH 7.5 at 18.0 mL (M_r 10 kDa) from the Superdex 200 column (Figure 5.5). This was significantly later than the expected elution volume for a protein of 105 kDa and implies that the toxin may be eluting as individual subunits. However, fractions of the peak run on an SDS-PAGE gel were found to contain all five PT subunits (results not shown). The untreated mutant PT sample was also analysed using a Superdex 75 column (Figure 5.5), which has an exclusion range suitable for resolving molecular weights of subunit size. Again the protein eluted as a single peak, at 10.6 mL (M_r 32 kDa), which had a slight trailing edge to ~18 mL (M_r 5.5 kDa), and there was no resolution of peaks corresponding to subunit size (Figure 5.5). Together these results illustrated that the untreated toxin was not eluting at the expected volume, possibly due to its different hydrodynamic volume in comparison to the molecular weight standards. Another possibility is that PT could have been retarded on the SEC columns possibly due to column interactions.

As can be seen from Figure 5.5, the 0.035 % FA-treated sample eluted at 16.0 mL (M_r 23 kDa) with a slight leading shoulder around 14 mL (M_r 57 kDa) and a trailing shoulder around 17 mL (M_r 15 kDa). With 0.1% FA-treatment the main peak eluted at 15 mL (M_r 37 kDa) with a leading shoulder again around 14 mL (M_r 57 kDa) and there was

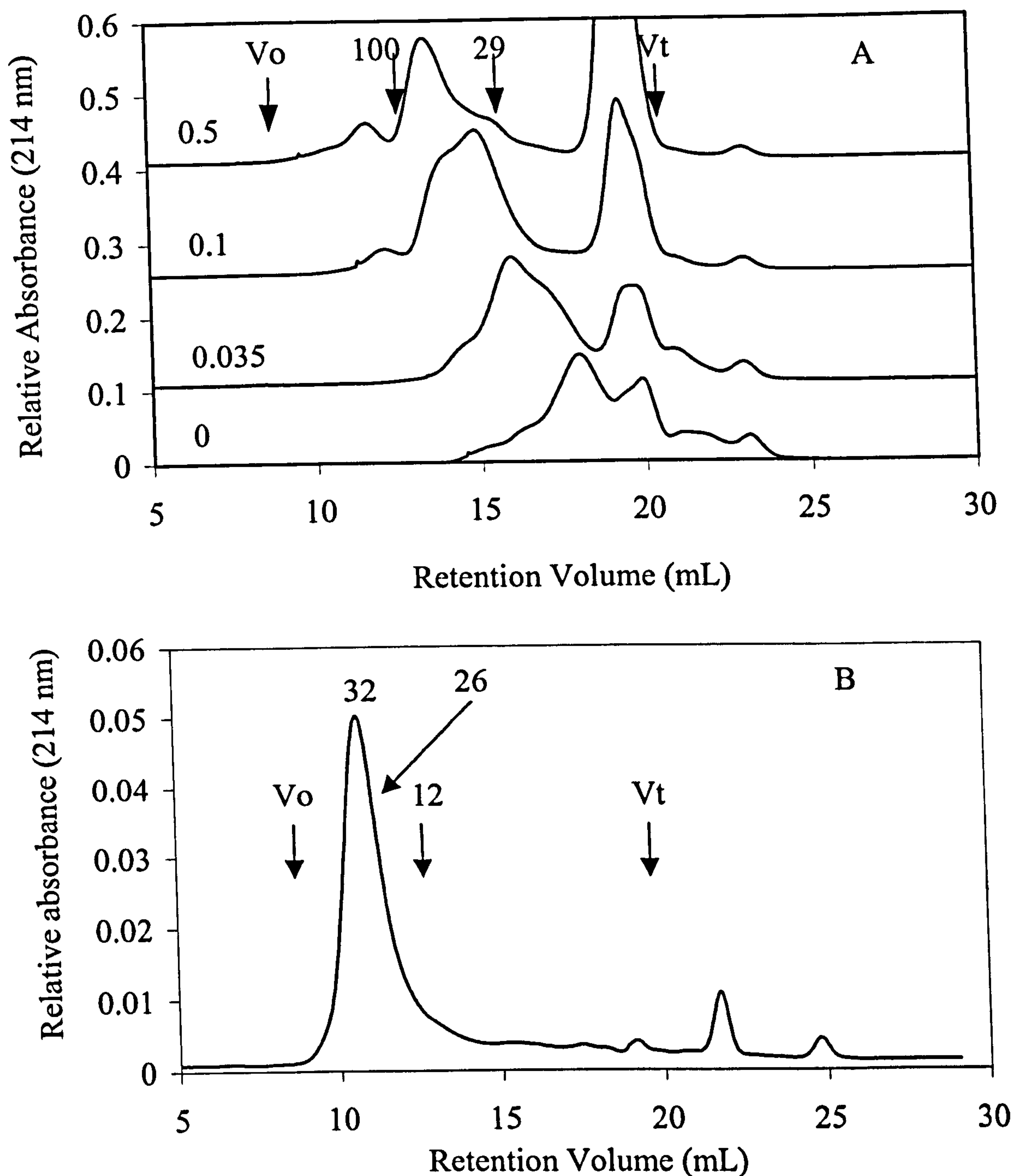


Figure 5.5 SEC/UV profiles of PT

Size exclusion chromatography with UV detection (SEC/UV) of mutant pertussis toxin (PT) preparations using an FPLC system equipped with a (A) Superdex 200 column, for analysis of untreated (under control conditions) and 0.035, 0.1 or 0.5 % (w/v) formaldehyde-treated samples or (B) Superdex 75 column for analysis of untreated mutant PT (not incubated). Running buffer was PBS, pH7.5, the samples applied were 15 μ g protein in 200 μ L PBS, pH 7.5. Elution positions of selected molecular weights (kDa) and of the exclusion volume (V_o) and total volume (V_t), as determined by calibration with protein standards, are indicated with arrows. Absorbance at 214 nm is shown.

development of an additional, low amplitude, high molecular weight peak at 12 mL (M_r 132 kDa). With 0.5 % FA there was an even earlier eluting peak at ~ 10 mL (M_r 300 kDa), followed by the peak at 12 mL (M_r 132 kDa) and main peak at 13.5 mL (M_r 72 kDa). Absorbance traces, at 280 nm, mirrored those obtained at 214 nm. Solution salts eluted as a large peak at 20 mL, just before the total volume of the column.

These results demonstrate that FA-treatment caused an increase in mutant PT relative molecular mass and an increase in the heterogeneity of mutant PT species. Estimates of molecular mass may not be accurate by this method since the untreated mutant PT holotoxin was shown to elute later than expected for its molecular mass. This may have been due to weak binding of the B-oligomer of PT to the polysaccharide moieties of the column. FA-induced changes in the B-oligomer may have prevented this association and thus cause the observed decrease in retention time. In addition, changes in conformation and charge could also be partly responsible for small shifts in elution time. However, the FA-dependent step-wise increases in molecular weight are of significance. Some of the elution volumes were common between preparations (Figure 5.5) demonstrating discrete increases in mutant PT molecular weight, as would be the case if certain subunits or multimers were associating or cross-linking to form favoured combinations. This is in agreement with the evidence of subunit cross-linking from SDS-PAGE. The largest species observed by SEC/UV had an estimated molecular mass of 300 kDa, which is nearly three times the mass of the holotoxin, indicative of multimeric structures larger than the limit of the SDS-PAGE gel. In order to confirm the presence of such large species these analyses were followed by SEC/MALLS.

SEC/MALLS of FA-treated PT

A combination of SEC coupled to refractive index and MALLS is a sensitive method for detection and more precise sizing of large molecules. This method obviates the need for column calibration with standards, which may not be similar in size and conformation to the sample. Mutant PT samples, treated with 0.035, 0.05 and 0.1 % FA were subjected to SEC/MALLS using an HPLC system equipped with two analytical columns (TSK G3000PW + TSK G4000PW), at a flow rate of 0.8 mL/min in PBS buffer, pH 7.5. Scattered light intensities of the eluted fractions were measured using a light scattering photometer and concentrations were measured using an interferometric refractometer. Twelve angles were used for measuring light scattering intensities and those at 90° are presented (Figure 5.6).

The low FA-treated sample (0.035 %) yielded a molecular mass of 367 kDa for peak 1, 121 kDa for peak 2, which was just slightly higher than the expected holotoxin molecular mass, and of ~7 kDa for peak 3 (Figure 5.6 and Table 5.1). Further FA treatment caused the molecular mass of these species to increase by amounts corresponding to holotoxin and dimeric and monomeric subunits. With 0.05 % FA the species in peak 1 increased to 423 kDa, in peak 2 increased to 140 kDa and in peak 3 increased to 66 kDa. Aggregates as large as 1 000 kDa were detected in the 0.1% FA sample.

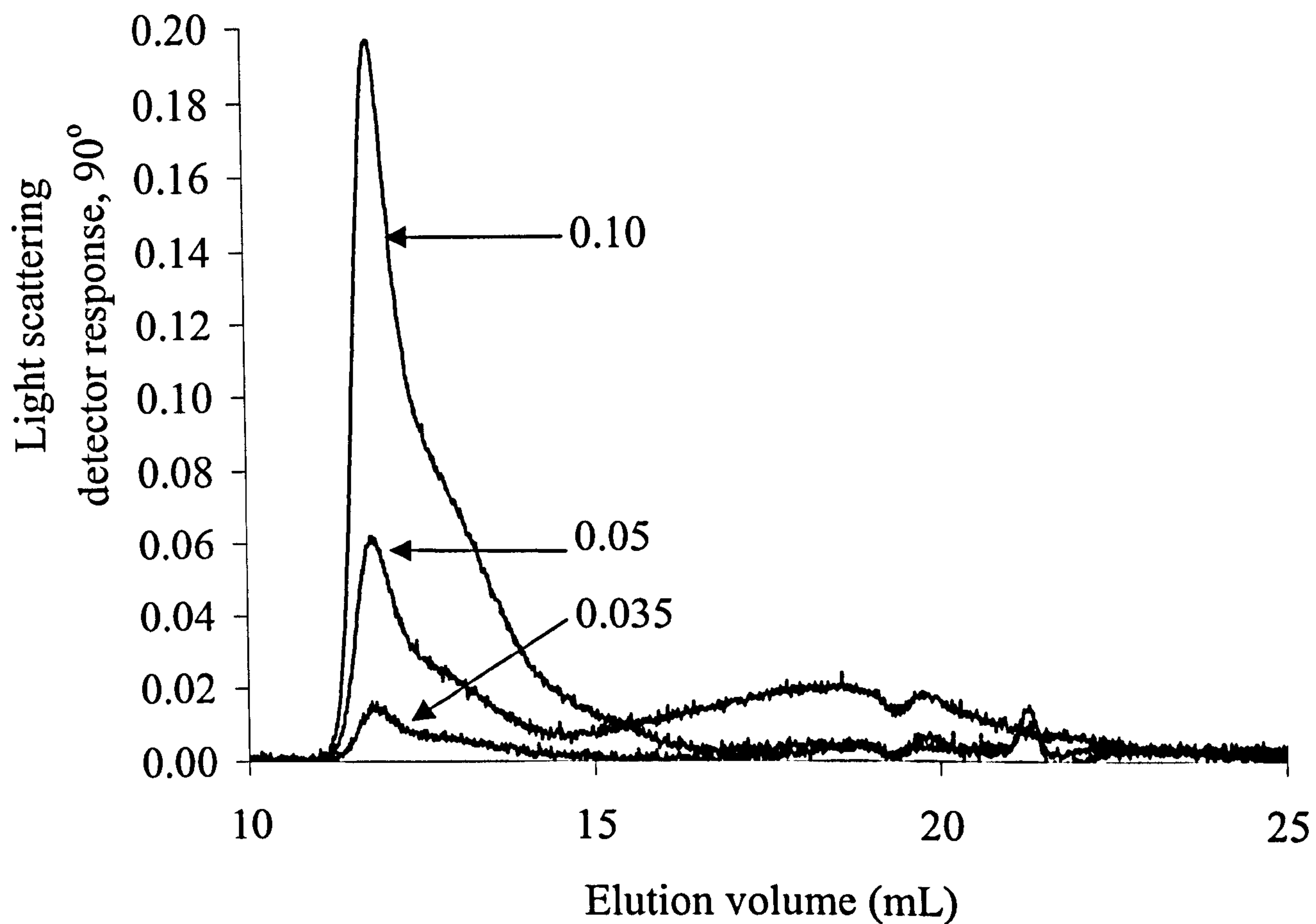


Figure 5.6 SEC/MALLS of FA-treated PT

Mutant pertussis toxin (PT) preparations ($\sim 500 \mu\text{g}$), which had been treated with 0.035, 0.05 and 0.1 % (w/v) formaldehyde (FA), were subjected to size exclusion chromatography (SEC) in PBS, pH 7.5, with detection by multi-angle laser light scattering (MALLS; 90° detector response shown) and refractive index (not shown) for determination of protein molecular masses (Table 1).

Table 5.1 SEC/MALLS of FA-treated PT

Mutant pertussis toxin (PT) preparations (~ 500 µg/mL), which had been treated with 0.035, 0.05 and 0.1 % (w/v) formaldehyde (FA) were subjected to size exclusion chromatography (SEC) in PBS, pH 7.5, with detection by multi-angle laser light scattering (MALLS; Figure 5.6) and refractive index monitoring (not shown). Molecular mass values are quoted for PT species eluting in the selected volumes. The error for each molecular mass estimate is associated with the statistical fluctuations in each detector signal.

PT sample, FA-treatment (%, w/v)	Peak elution (mL)	Molecular mass (kDa)
0.035	11.32-12.33	Peak 1: 367.1 ± 60
	12.43-14.89	Peak 2: 120.7 ± 30
	17.36-20.59	Peak 3: 6.7 ± 3
0.05	11.26-12.44	Peak 1: 423.3 ± 30
	12.53-14.28	Peak 2: 140.3 ± 20
	14.79-18.70	Peak 3: 65.8 ± 9
	19.03-20.21	Peak 4: 5.4 ± 1
0.1	11.31-12.49	Peak 1: 1004.0 ± 30
	12.63-14.09	Peak 2: 270.0 ± 8
	14.23-14.79	Peak 3: 91.9 ± 10
	14.89-15.78	Peak 4: 48.6 ± 10

These results support those from SEC /UV and in addition give an indication of the sizes of the very largest species, which were not detected by UV-detection possibly because they were present in very small concentrations.

AUC of FA-treated mutant PT

AUC was used in order to analyse the protein in solution, rather than associated with a gel matrix. Mutant PT preparations, untreated and treated with 0.05 and 0.1 % FA were analysed by AUC to determine their equilibrium solute distributions. Three different concentrations of each sample were analysed in PBS dialysate, pH 7.4. Molecular weight distributions were recorded with scanning absorbance at three rotor speeds, 15 000, 25 000 and 35 000 rpm. A true optical baseline was determined by sedimenting the protein from the bulk of the solution volume at 47 000 rpm. Using the MSTAR programme (Cölfen and Harding, 1997) the value of M^* at the cell base, which is equal to the apparent weight average molecular weight ($M_{w,app}$) (kDa) of the whole sample, was calculated for each concentration of mutant PT preparation at each rotor speed (Figure 5.7 A).

The $M_{w,app}$ of untreated mutant PT at low rotor speed (15 000 rpm) was ~ 70 kDa (Figure 5.7 A). This decreased to ~ 20 kDa at 35 000 rpm. Since the observed molecular weight represents the average across the radial distribution of sample solute, these results suggest that mutant PT exists in solution partly as unattached B-oligomer (79 kDa) and A-subunit (26 kDa), which have markedly different equilibrium distributions owing to their

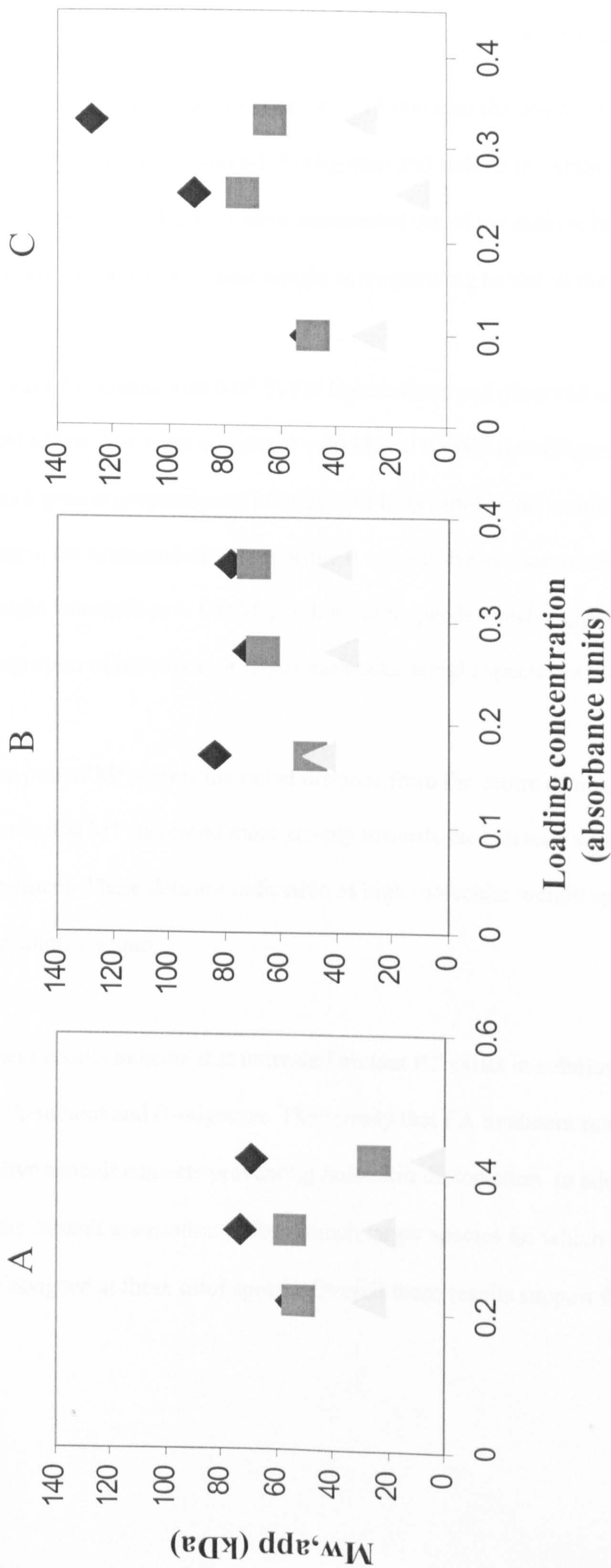


Figure 5.7 (A) AUC analysis of FA-treated PT

Mutant pertussis toxin (PT) preparations in PBS, pH 7.5, which had been treated with 0 (A), 0.05 (B) and 0.1 (C) % (w/v) formaldehyde (FA) were subjected to sedimentation equilibrium ultracentrifugation (AUC). Molecular weight distributions of samples at three different concentrations (determined at 271 nm, with a 1.2 cm pathlength) were analysed at three rotor speeds: 15 000 (diamonds), 25 000 (squares) and 35 000 (triangles) rpm. The absorbance distribution of each sample at each rotor speed was corrected by subtracting the buffer baseline (absorbance at 47 000 rpm) and analysed by the model-independent programme, MSTAR (Cölfen and Harding, 1997), to determine apparent weight average molecular weights ($M_{w,app}$).

different molecular masses. That is, at 15 000 rpm the observed molecular weight is an average of that for A-subunit, B-oligomer and holotoxin, while at 35 000 rpm the holotoxin and B-oligomer have sedimented out of the main solute column giving an average observed molecular weight corresponding to that of the A-subunit.

Mutant PT treated with 0.05 % FA had an increased observed molecular weight of ~ 80 kDa at low rotor speed and ~ 40 kDa at 35 000 rpm (Figure 5.7 A), suggesting there was a greater proportion of holotoxin or larger molecular weight species in this sample than in the untreated sample. For the 0.1 % FA-treated sample the observed molecular weight increased to ~ 100 kDa at low rotor speeds which suggests an even larger proportion of holotoxin or larger molecular weight species in this preparation.

The plot of M^* versus the radial distance from the centre of rotation (Figure 5.7 B), shows that M^* increased more steeply towards the cell base with increasing FA treatment. These data are indicative of high molecular weight species sedimenting out of the solute column.

These results indicate that untreated mutant PT exists in solution partly dissociated into its A-subunit and B-oligomer. They imply that FA treatment reinforces the association of native subunit contacts preventing holotoxin dissociation. In addition there is further inter-subunit association to form much larger species for which molecular weights cannot be assigned at these rotor speeds. Overall these results support the data from the three

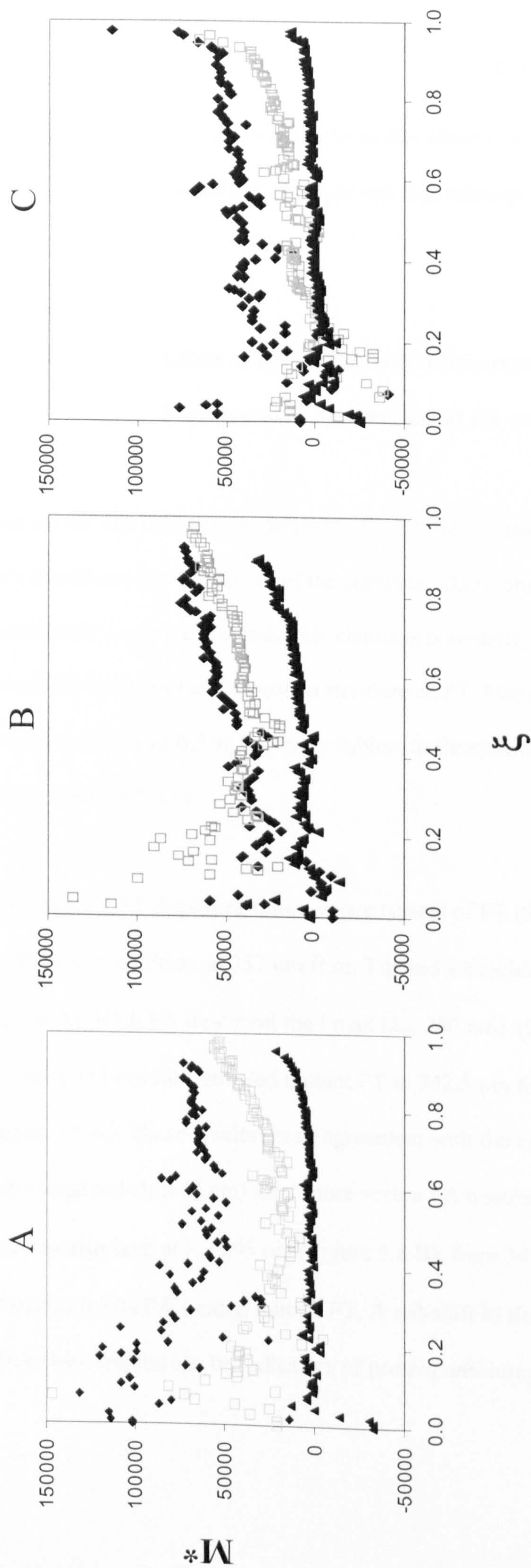


Figure 5.7 (B) AUC analysis of FA-treated PT

Mutant pertussis toxin (PT) preparations, treated with (A) 0, (B) 0.05 and (C) 0.1 % (w/v) FA, of absorbances ($A_{271\text{ nm}}$, 1.2 cm) 0.32, 0.36 and 0.25 respectively, were subjected to sedimentation equilibrium analytical ultracentrifugation (AUC) at three rotor speeds: 15 000 (diamonds), 25 000 (squares) and 35 000 (triangles) rpm as described in Figure 5.7A. M^* distributions are shown. ξ is the normalised radial distance from the centre of rotation. M^* at the cell base, i.e. at $\xi = 1$, represents the apparent weight average molecular weight over the whole distribution of sample solute. Data close to the meniscus (i.e. $\xi = 0$) are noisy, giving some irrational negative values. These are not taken into account in extrapolation, which is to the cell base (i.e. $\xi = 1$), for the determination of weight average molecular weights ($M_{w,app}$).

other sizing methods. In order to characterise the effect of FA on the molecular conformation of PT fluorescence spectroscopy was subsequently performed.

5.2.3

Effect of FA on the molecular conformation of PT

Fluorescence spectroscopy of FA-treated PT

PT contains six Trp residues distributed in the S1, S2, S3 and S5 subunits and also 62 Tyr residues distributed throughout all of the subunits. Therefore, intrinsic fluorescence of the environmentally sensitive aromatic side chains is potentially a sensitive indicator of changes in the local, molecular protein structure of PT. Mutant PT, untreated and treated with 0.035, 0.05, 0.1 or 0.5 % FA, were subject to fluorescence spectroscopy as described previously (section 5.2.1).

Untreated mutant PT displayed fluorescence typical of PT (Seabrook, Atkinson, and Irons, 1991) with an Fmax at 337 nm from Trp and a shoulder at ~ 305 nm from Tyr (Figure 5.8 A). With FA treatment the Fmax (λ_{ex} 280 nm) of mutant PT gradually red-shifted, from 337 nm for untreated mutant PT to 342.5 nm for 0.5 % FA-treated mutant PT (Figure 5.8 A). These results are in agreement with the experiment above, which showed a large red-shift (7 nm) after more severe FA treatment (Figure 5.2). A red-shift of 3 nm was also seen at λ_{ex} 295 nm (Figure 5.8 B), from 347 nm for untreated mutant PT to 350 nm for 0.5 % FA-treated mutant PT. A red-shift in fluorescence at both of these excitation wavelengths can be indicative of protein unfolding.

The emission spectra (λ_{ex} 280 nm) also changed shape with FA-treatment due to changes in the relative intensities of Trp and Tyr fluorescence (Figure 5.8 A). Normalising the spectra at 305 nm (not shown) exemplifies the gradual increase in Trp fluorescence with respect to Tyr as FA-treatment increased. Taken together with the red-shifts, these changes in Trp intensity are indicative of a change in protein folding.

It was also possible that the formation of Trp derivatives was responsible for the modified fluorescence emission of mutant PT. Trp is accessible to N-hydroxymethylation by formaldehyde and can undergo cross-linking reactions with other side-chains (Tome and Naulet, 1981; Kitamoto and Maeda, 1980; Means and Feeney 1971a). Known Trp derivatives, which may be formed by reactions with FA, tend to absorb light at wavelengths longer than those absorbed by native Trp. The tryptophan derivatives, norharman and kynurenine, have maximal absorbance in the range 315 - 375 nm and corresponding maximal fluorescence in the range 440 – 490 nm (Udenfriend, 1962; Morton, 1975). Neither absorbance nor fluorescence at these wavelengths was observed for any of the mutant PT preparations suggesting that such modifications were not responsible for the observed fluorescence changes with increasing FA. We conclude that the red-shifts and increasing Trp intensity observed are most likely due to increasing exposure of Trps to solvent due to protein unfolding.

Fluorescence spectroscopy of FA-treated PT SEC fractions

Mutant PT, untreated and treated with 0.5 % FA, were subjected to SEC using an FPLC

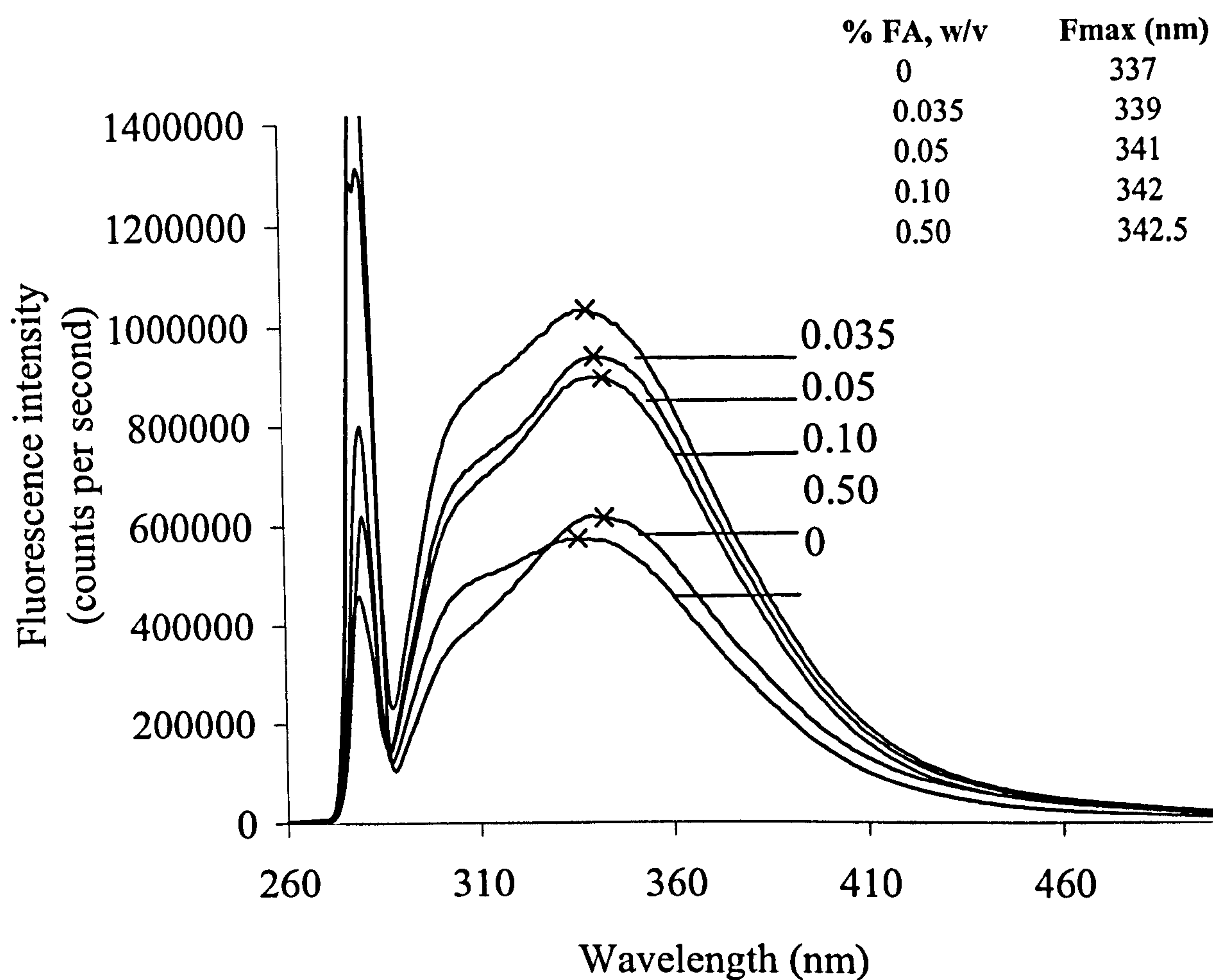


Figure 5.8 (A) Intrinsic fluorescence of PT treated with different concentrations of formaldehyde (λ_{ex} 280 nm)

Mutant pertussis toxin (PT) samples (10 - 30 $\mu\text{g/mL}$) in PBS, pH 7.5, which had been treated with 0, 0.035, 0.05, 0.1 and 0.5 % (w/v) formaldehyde (FA), were excited at 280 nm, their fluorescence scanned from 260 - 500 nm, and spectra corrected by subtraction of the PBS base-line spectra.

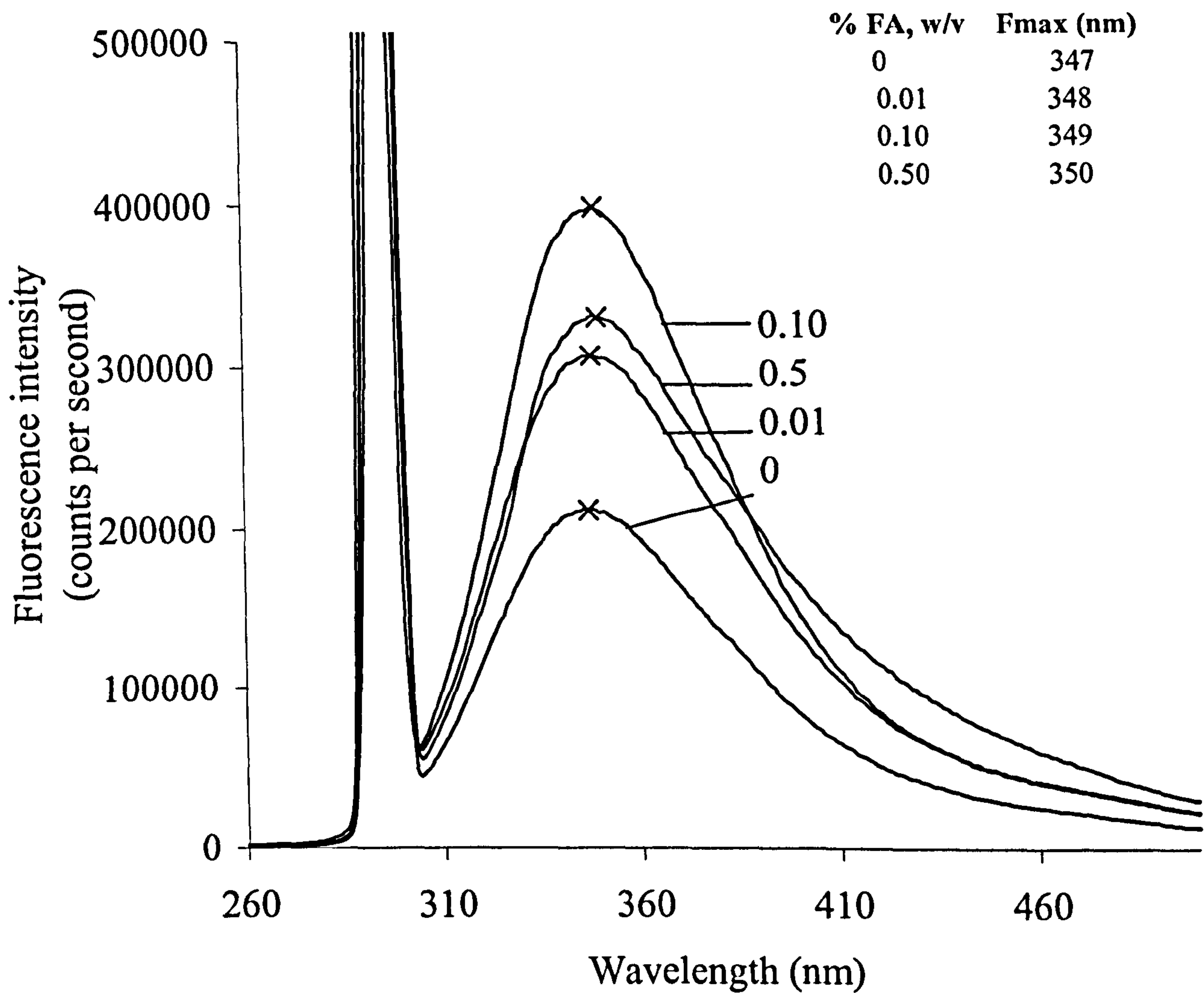


Figure 5.8 (B) Intrinsic fluorescence of PT treated with different concentrations of formaldehyde (λ_{ex} 295 nm)

Mutant pertussis toxin (PT) samples ($\sim 50 \mu\text{g/mL}$) in PBS, pH 7.5, which had been treated with 0, 0.01, 0.10 and 0.50 (w/v) formaldehyde (FA), were excited at 295 nm, their fluorescence scanned from 260 - 500 nm, and spectra corrected by subtraction of the PBS base-line spectra.

system equipped with a Superdex 200 column, as described above, and 0.5 mL fractions were collected. For each preparation, fractions of the same peak were pooled and fluorescence measurements taken.

It can be seen that the main SEC peak of untreated mutant PT had an F_{\max} of 336 – 337 nm (λ_{ex} 280 nm) (Figure 5.9), which is typical of whole non-fractionated PT (Figure 5.8 A). The latest-eluting fraction of the 0.5 % FA-treated sample had an F_{\max} of 339 nm, which is significantly red-shifted compared to untreated whole PT. Increasingly earlier eluting fractions of 0.5 % FA-treated mutant PT exhibited decreasingly red-shifted F_{\max} values. The highest molecular weight species (F_{\max} , 336 nm) is likely to be an aggregated version consequently with more buried Trps.

Together these findings indicate that FA is altering the native folding of the protein initially causing unfolding followed by re-association of the non-native species to form aggregates leading to an increase in molecular weight.

5.3 Discussion

The ultimate goal in vaccine development is to produce a non-toxic immunologically efficacious product. The use of FA in the detoxification of bacterial toxins to produce vaccine components is cheaper and simpler to employ than protein-engineering methods and is still used by manufacturers of tetanus, diphtheria and pertussis vaccines worldwide. Moreover, non-toxic antigens and genetically inactivated proteins often

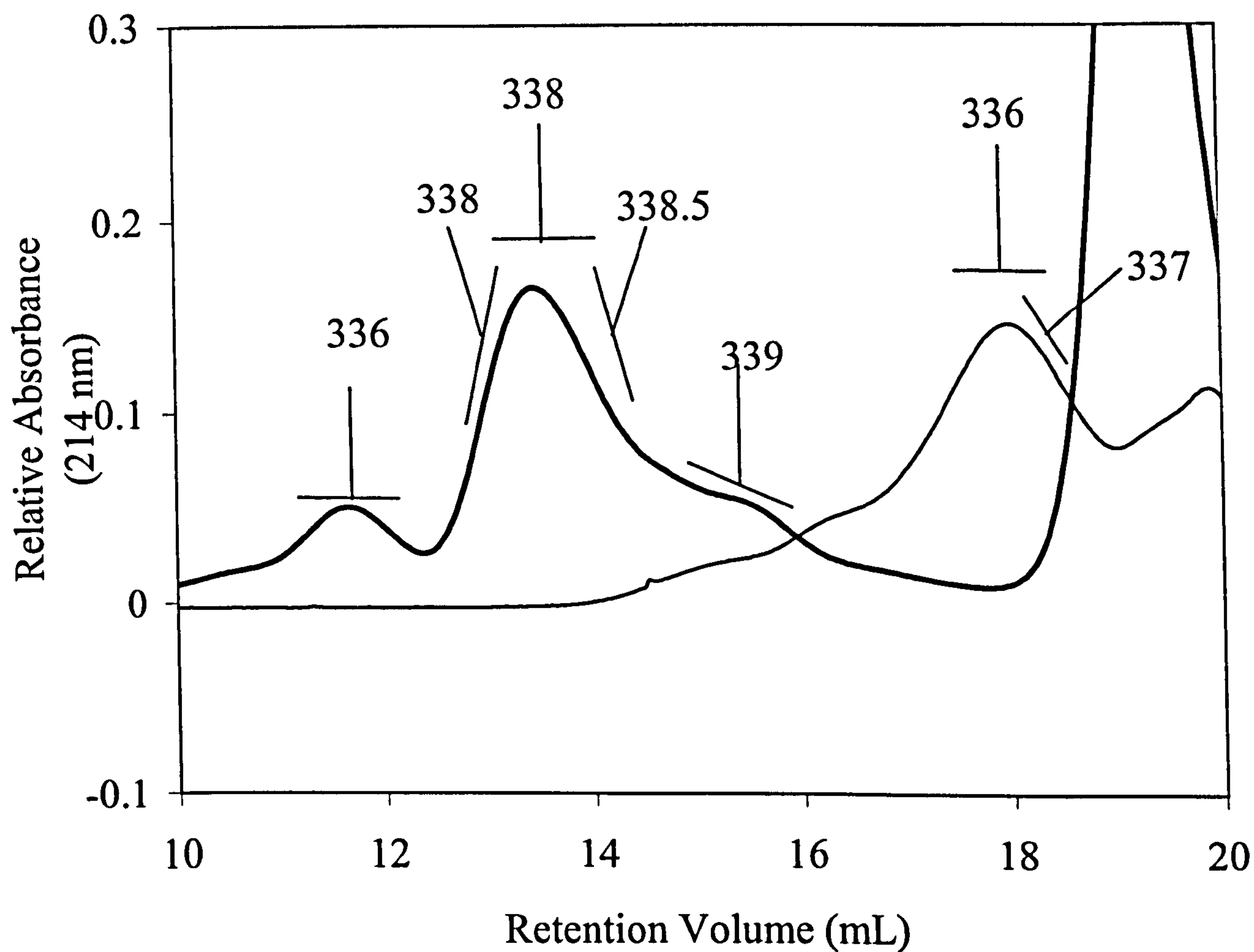


Figure 5.9 Intrinsic fluorescence analysis of SEC-fractionated PT
Mutant pertussis toxin (PT) preparations (15 μ g PT in 200 μ L PBS, pH 7.5), untreated (faint trace) and 0.5 % (w/v) formaldehyde (FA)-treated (bold trace), were subjected to size exclusion chromatography (SEC) using an FPLC system with a Superdex 200 column, and eluted in PBS with detection at 214 nm. Column fractions, indicated by the T-bars, were analysed by fluorescence spectroscopy (λ_{ex} 280 nm) and for each fraction the Fmax values (nm) are labelled.

require FA treatment for stabilisation (Nencioni *et al.*, 1990; Petre *et al.*, 1996). There is a need to characterise the heterogeneous mixtures that can be produced by formaldehyde.

The application of biochemical and biophysical techniques is enabling the manufacture of products that are increasingly well-defined in terms of purity, component size and molecular association.

Primarily, it was shown by SEC and fluorescence that mutant and native PT have the same structural characteristics and are modified in the same way with FA treatment.

Untreated and FA-treated mutant PT preparations were then analysed in more detail using a variety of sizing techniques to characterise the heterogeneous species that could arise from FA treatment of an oligomeric protein. AUC provided evidence that untreated mutant PT exists in solution as a proportion of holotoxin, B-oligomer and A-subunit. All of the sizing techniques gave complementary evidence to support the conclusions that low FA treatment of mutant PT stabilises native subunit contacts and further FA treatment causes additional subunit association, cross-linking and aggregation. Some of the mechanisms involved in this association can now be discussed in detail.

Untreated PT

The AUC results suggest that mutant PT in solution is made up of a proportion of holotoxin, A-subunit and B-oligomer. It is known that full activation of PT holotoxin requires binding of ATP to the B-oligomer to destabilise the A-subunit and B-oligomer (Moss *et al.*, 1986; Burns and Manclark, 1986). Residual activity has been found in PT without ATP added (Moss *et al.*, 1986; Burns and Manclark, 1986) also suggesting that

PT exists partially as separate A and B domains. There may be an equilibrium between the associated and non-associated states. Comparison of the crystal structures of PT before and after ATP binding, which is distant from the S1/B-oligomer interface, shows that there are only minor side-chain movements and no extensive changes in the protein backbone (Hazes *et al.*, 1996). It may be that this is enough to switch the equilibrium to the non-associated state. It was suggested that the triphosphate moiety of ATP may sterically repel the C terminus of the S1 subunit, thus dissociating it from the B-oligomer (Hazes *et al.*, 1996; Kaslow *et al.*, 1987). It is feasible that this mechanism does not operate by 'pushing' the S1 subunit out of the B-oligomer pore, but by the ATP molecules occupying the free B-oligomer population and preventing their re-association with S1 subunits.

FA-treated PT

Cross-linking

The results of SDS-PAGE, SEC-MALLS and AUC collectively substantiate the hypothesis that, with small concentrations of FA, covalent cross-linking occurs between amino acids on neighbouring subunits causing an increase the number of species of holotoxin size, perhaps by preventing subunit dissociation. This study also demonstrates that higher FA treatment produces molecules exceeding holotoxin molecular weight providing novel evidence of cross-linking between holotoxins or large multimeric species. Studies on FA-treatment of the oligomeric VacA protein, of *Helicobacter pylori*, have provided some evidence, from SDS-PAGE, of covalent, inter-monomer cross-linking (Manetti *et al.*, 1997). Presumably intra-subunit cross-links also occur. Following

FA-treatment, the S1-subunit band on SDS-PAGE gels is preserved for longer periods of incubation at 37 °C (Nencioni *et al.*, 1991). These findings are in accordance with the known cross-linking reactions of FA with amino acids.

In reaction with FA, certain amino acids become modified forming a reactive hydroxymethyl derivative. Hydroxymethylated amino acids can react in a variety of ways, with each other or with non-modified amino acids, to form different chemical cross-links. FA cross-links were analysed in FA-treated bovine serum albumin using nuclear magnetic resonance (NMR) spectroscopy (Tome, Kozlowski, and Mabon, 1985). Lys, the most FA-reactive amino acid, in solution can link to protein amino acids via its hydroxymethylated epsilon amino group to form a methylene bridge (Tome and Naulet, 1981) (Tome, Kozlowski, and Mabon, 1985). These were found to be primarily between lysine and arginine, asparagine, glutamine or tyrosine, via their reactive hydroxymethyl derivatives. Secondary reactions between amino acids other than lysine, namely, tyrosine, tryptophan, histidine, asparagine and glutamine were also implicated (Tome, Kozlowski, and Mabon, 1985). In addition, amino acid derivatives such as methyllysine and formyllysine were detected. These FA products can be divided into three types: stable, acid labile or reversible (Bizzini and Raynaud, 1974; Tome *et al.*, 1979).

The laddering and smearing effect on SDS-PAGE gels, above 0.035 % (w/v) FA, is likely to be due to various side-chain modifications, including decoration of the protein with lysine from the reaction mixture. Free lysine linked to the protein via its hydroxymethylated epsilon amino group (Tome and Naulet, 1981; Tome, Kozlowski, and

Mabon, 1985) has an exposed protonated alpha amino group. FA treatment of pertactin protein from *B. pertussis* showed that increasing the lysine concentration caused an increase in the isoelectric point of the protein (Ceccarini *et al.*, 2000), in accordance with our proposal of lysine decoration. Moreover, previous results from amino acid analysis of FA-treated PT, found an increase in the lysine content of PT (Nencioni *et al.*, 1990). In order to confirm specific subunit-subunit cross-linking and further characterize the subunit composition of FA-induced multimeric structures, Western blotting using subunit specific mAbs was subsequently performed and these results are described in the next chapter (Chapter 6).

Unfolding

The red-shifts in fluorescence spectroscopy with increasing FA-treatment suggest that FA also causes an increase in the unfolding of mutant and native PT. These results agree with a previous study which showed a significant red-shift in the fluorescence spectra of aldehyde-treated PT (Bolgiano *et al.*, 1999). Unfolding occurs regardless of the covalent cross-links. Unfolding may be due to intra-molecular charge effects caused by FA, intra-molecular lysine additions and/or inter-molecular covalent cross-links or other interactions disrupting and opening up the native, and methylene cross-linked, subunit structures. The largest species appear to have masked, Trps compared to the bulk of the sample suggesting that they are formed by aggregation of the unfolded material. A series of electron micrographs of FA-treated VacA protein illustrate the loss of its shape and definition during the formation of aggregates (Manetti *et al.*, 1997).

Oligomerisation and aggregation

The high molecular weight species detected by SEC/UV, SEC/MALLS, AUC and SDS-PAGE could represent specifically associated subunits or holotoxins (oligomers), or less uniform association of subunits of non-native structure (aggregates). Since oligomers and aggregates could not be absolutely differentiated in this study, the high molecular weight species are generally referred to as aggregates. Aggregates as large as 1000 kDa were observed by SEC/MALLS. Species up to 900 kDa have been previously observed in a commercial pertussis toxoid (Bolgiano *et al.*, 1999).

A proportion of the aggregates was comprised of stable structures, as confirmed by their detection with SDS-PAGE. In addition, there was evidence of some dissociation, suggesting that less stable structures were also formed. In the 0.035 % FA treated preparation, large species exceeding 100 kDa were detected by SEC/MALLS, while species only as large as 50 kDa were detected with SDS-PAGE.

Taken together, the results demonstrated that stable and reversible interactions are involved in subunit association, oligomerisation and aggregation of PT. This is in line with the known cross-linking reactions of FA (see above; *Cross-linking*). In addition the amino acid derivatives produced are likely to change the charge and hydrophobicity of the molecule, which could facilitate non-covalent associations between subunits. The next chapter (Chapter 6) will assess the integrity of the epitopes in these cross-linked species.

Immunological implications

It is known that protection against *B. pertussis* is dependent on both antibody specificity and cell-mediated immunity (Mahon *et al.*, 1997; Redhead *et al.*, 1993; Mills *et al.*, 1993; Ryan *et al.*, 1998a; Mahon *et al.*, 1996). Therefore structural changes in the immunogenic PT molecule, including changes in its conformation, charge and cross-linking, which could alter antigen processing or surface structural epitopes, are of significance.

Physico-chemical methods have provided a detailed description of the molecular forms comprising FA-treated PT, and now their significance in the immune response can start to be realised. Subunit cross-linking and epitope integrity will be studied in the next chapter (Chapter 6). The immune response to FA-treated mutant and native PT will also be assessed. Obtaining structural correlates of protection and developing reliable alternatives to animal experiments are priorities in the pertussis field. Detailed physico-chemical characterisation of PT and other vaccine components will drive the development of better-defined and potentially more immunogenic vaccines.

6.1

Introduction

FA was the one of the first chemicals accepted for the detoxification of vaccine products, and is still used to toxoid bacterial protein toxins for use in vaccines licensed around the world. FA-detoxified PT is commonly used in acellular pertussis vaccines. However, the effects of FA treatment on the immunological properties of the antigen are poorly understood. There have been no direct studies on the effect of FA on the immunology of native PT to our knowledge, and only limited studies on mutant PT (PT-9K/129G) have been reported (Nencioni *et al.*, 1990; Cropley *et al.*, 1995; Di Tommaso *et al.*, 1994; Nencioni *et al.*, 1991).

Mutant PT is stabilised with a relatively low concentration of FA (0.035 %, w/v) for use in one of the acellular pertussis vaccines. In order to decide the stabilising concentration of FA, some immunological studies were performed on a series of FA-treated mutant PT preparations. The effect of FA on anti-PT antibody recognition of mutant PT epitopes was assessed, using competitive radioimmunoassay, and it was shown that the affinity of polyclonal anti-PT serum for mutant PT was reduced by 16-fold with 0.21 % (w/v) FA (Nencioni *et al.*, 1991). In addition, the affinity of anti-S1 mAb, 1B7, for mutant PT was reduced to undetectable levels with 0.052 % (w/v) FA-treatment (Nencioni *et al.*, 1991). However, in the same study, screening with the 1B7 mAb in Western blotting generated

dense S1 bands for even the highest FA-treated sample (0.42 %, w/v, FA). It was suggested that the 1B7 epitope may have been masked in solution and unmasked with the denaturing technique of Western blotting.

T-cell epitope studies showed that recognition of mutant PT by T-cell clones specific for the wild-type S1 subunit was not affected by treatment of mutant PT with 0.42 % (w/v) FA (Nencioni *et al.*, 1991). Another study showed that a much higher concentration, of 3.5 % (w/v) FA, destroyed the ability of mutant PT to induce proliferation of S1-specific T-cell clones (Di Tommaso *et al.*, 1994).

The effect of FA on the immunogenicity of mutant PT has also been researched. Serum was raised in guinea pigs, against the 0 – 0.42 % (w/v) FA-treated mutant PT preparations, and were analysed by ELISA for levels of IgG antibodies specific for PT holotoxin or B-oligomer (Nencioni *et al.*, 1991). The level of PT-specific IgG antibody was not significantly affected by FA-treatment of the mutant PT antigen. However, the B-oligomer-specific IgG response progressively increased with increasing FA-treatment (0 – 0.42 %, w/v). This study also found a decrease in the serum neutralizing titre with FA-treatment of mutant PT, as determined by CHO-cell assay, which corresponded with the increase in B-oligomer specific IgG antibodies. They concluded that the overall level of PT-specific IgG antibody response is not affected by FA-treatment but that the fine specificity and neutralising properties of the antibodies obtained are altered.

Such changes in the immunogenicity between FA-treated PT toxoids may contribute to the differences observed in the immunogenicity of acellular pertussis vaccines. For example, it has been shown that different acellular vaccines induce Th1 and Th2 responses to different extents (Ausiello *et al.*, 1997; Mills *et al.*, 1998; Ryan *et al.*, 1998a). However, the effect of FA on the immunogenicity of both native and mutant PT, used in acellular pertussis vaccines, has not yet been fully characterised.

In this chapter, the aim was to study the effect of a series of FA concentrations on the immunogenic properties of both mutant and native PT. The effect of FA on the epitope characteristics and on the PT-specific murine antibody response was assessed. This study design permitted the novel investigation of the immunogenic effect of FA on the toxic native PT with respect to the catalytically inactive mutant PT.

6.2

Results

Mutant and native PT treated with 0 – 1.0 % (w/v) FA (*High FA-treated PT*, section 2.2.3), which covers the concentration range used in commercial toxoiding, were used throughout this study.

6.2.1 Characterisation of PT subunit cross-linking and epitope integrity after FA-treatment, by immunoassay

The effect of 0 – 0.1 % FA on PT epitopes was analysed using PT subunit-specific mAbs to screen PT preparations in ELISA and immunoblotting. ELISA was performed to quantitatively assess the subunit epitopes, and immunoblotting was chosen to provide additional information on the subunit cross-linking of the antigen preparations.

PT epitope recognition by murine mAbs in ELISA assay

In the ELISA analysis, 96-well plates were coated with untreated and 0.1 % FA-treated, mutant and native PT preparations and an in-house reference PT (P57) in duplicate, and then screened with mAbs specific for subunits S1 – S5. The geometric mean potencies of mAb binding to each preparation were calculated by parallel line analysis relative to the reference PT.

All of the subunit-specific mAbs showed good binding to both untreated native and mutant PT (Figure 6.1). Binding of the anti-S1, S4 and S5 mAbs to both native and mutant PT treated with 0.1 % FA was significantly lower, by 50 to 100 % ($P \leq 0.1$), compared with the untreated antigens (Figure 6.1). After 0.1 % FA-treatment, there was a significantly greater decrease in the S1-specific mAb binding to native PT (59 % decrease) than to mutant PT (44 % decrease) ($P \leq 0.1$). There was also a significantly greater decrease in the S5-specific mAb binding to native PT (100 % decrease) compared with mutant PT (83 % decrease) ($P \leq 0.1$) after 0.1 % FA-treatment of the antigens. For

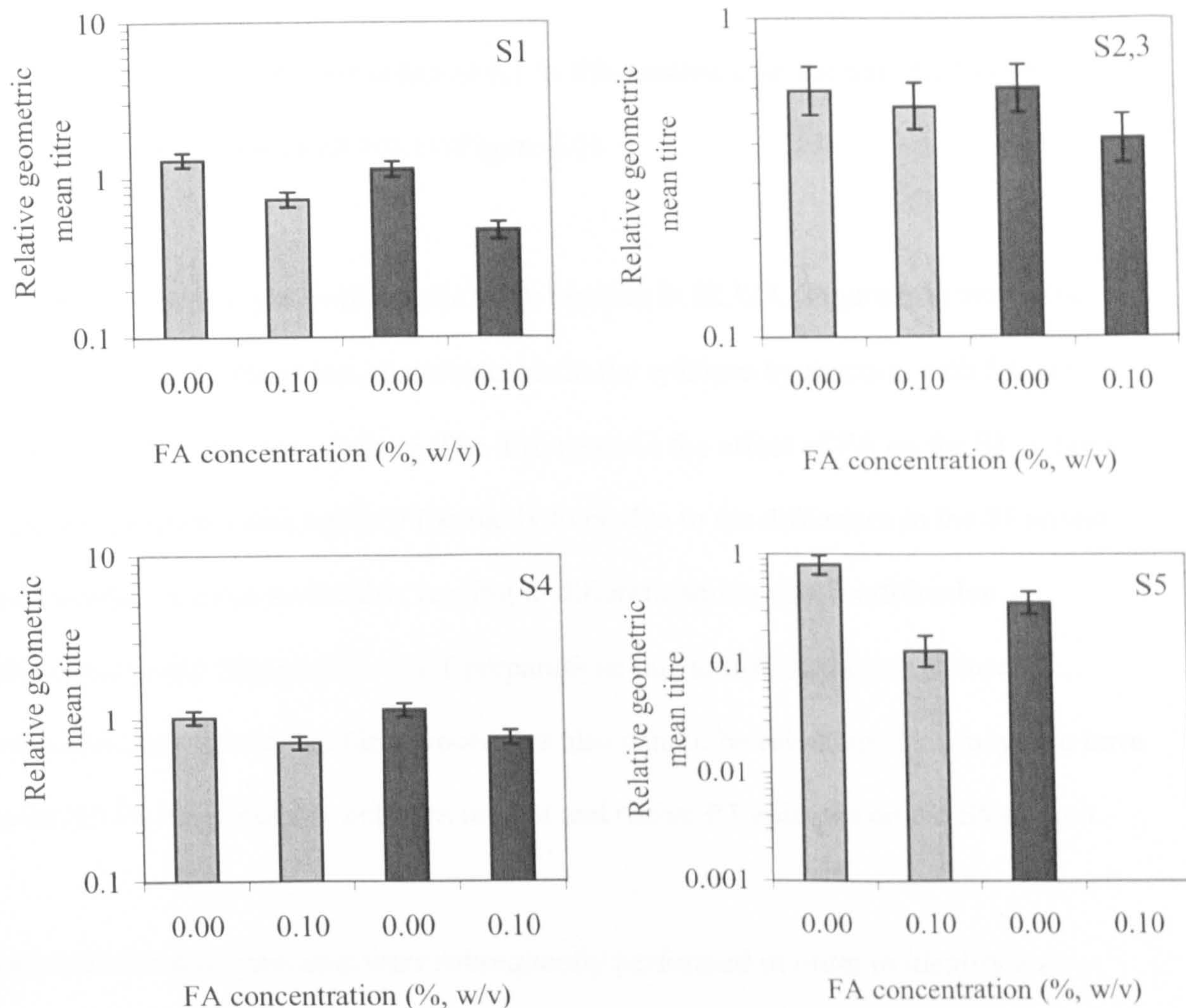


Figure 6.1 The effect of FA on PT epitopes as determined by mAb-binding

Mutant (grey bars) and native (black bars) pertussis toxin (PT), treated with 0 and 0.1 % (w/v) formaldehyde (FA) , were used to coat 96 well plates and screened with monoclonal antibodies (mAbs) specific for epitopes on each of the five subunits (S1-S5). One of the mAbs used recognised both S2 and S3. Mab titres were calculated relative to a PT reference and expressed as a mean of three repeat experiments, with 95% confidence limits denoted by error bars. These results are representative of two separate experiments.

* The potency for anti-S5 binding to 0.1 % FA-treated native PT was undetectable.

both mutant and native PT, the effect of 0.1 % FA-treatment on the anti-S2/3 mAb binding was not significant ($P > 0.1$) (Figure 6.1).

The reduction in epitopes available for mAb binding in ELISA (Figure 6.1) may have been due to specific alteration of amino acids in the epitopes by reaction with FA and lysine, such as lysine cross-linking. The difference in the effect of FA on the S1 subunit of mutant compared with native PT is likely to be due to the difference in the S1 amino acid sequence of these molecules, leading to different amino acid modification.

Differences in stability of the two PT preparations due to different manufacturers' production, storage and handling procedures also cannot be ruled out. This may also have accounted for the variations between mutant and native PT epitopes on the S5 subunit.

Immunoblotting experiments were subsequently performed in order to identify any subunit epitopes in the high molecular weight species detected in SEC and SDS-PAGE (Chapter 5) and to characterise the inter-subunit covalent cross-linking. In addition, this technique was used for comparison with ELISA, and to address the possibility of epitope depletion via masking within reversibly associated complexes.

PT epitope recognition by murine mAbs in immunoblotting assay

Untreated and 0.035, 0.05 and 0.1 % FA-treated mutant PT preparations were separated by SDS-PAGE and blotted onto a nitrocellulose membrane. Each membrane was screened with one of the five mAbs specific for subunits S1 – S5.

The five immunoblots (Figure 6.2 A), with the aid of the summary plot (Figure 6.2 B), illustrate the typical bands corresponding to the five subunits of untreated mutant PT (11 – 26 kDa). With FA treatment up to 0.1 %, all monomeric subunits were still detected and in addition combinations of subunits were detected in higher molecular weight bands (Figure 6.2 A and B).

The first high molecular weight band was a distinct band detected at M_r 37 – 45 kDa in all of the FA-treated mutant PT samples by all five subunit-specific mAbs. This could correspond to a combination of dimeric and trimeric cross-linked subunits. Initially these are likely to be S2/3 + S4 (M_r 34 kDa) and/or S2/3 + S4 + S5 (M_r 45 kDa), since S2 – S4 and S3 – S4 dimers have the most extensive native interactions of the PT molecule (Stein *et al.*, 1994a). Cross-linked S1 + S5 (M_r 37 kDa) were also likely to have existed in this band. With FA-treatment above 0.035 % FA, another band was detected at M_r 57 - 65 kDa, by all of the mAbs, and is likely to correspond to the cross-linking of another dimeric subunit or of another subunit, perhaps S1 or S5, to the dimeric and trimeric complexes.

In samples treated with more than 0.035 % FA, bands of higher molecular weight, above $\sim M_r$ 60 kDa were observed. These high molecular weight bands were more frequent, broader and were joined by smears. Broad bands indicate that protein species were no longer homogeneous. In this case they may suggest that there was an array of lysine-decorated, and perhaps differently modified proteins, which could still be recognised by

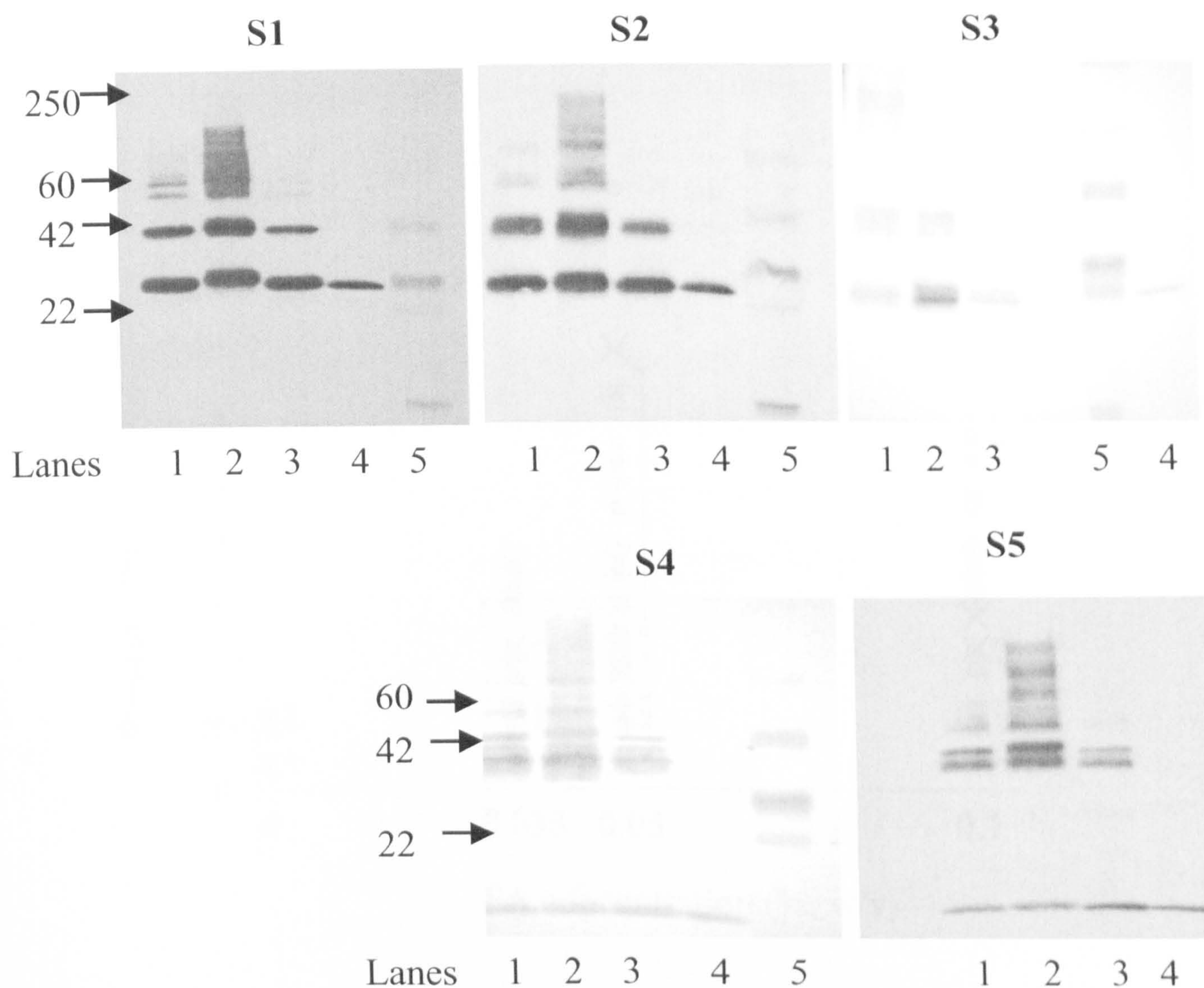


Figure 6.2 (A) Effect of FA on PT subunit cross-linking

Mutant pertussis toxin (PT) samples (5 μ g) treated with 0 (Lane 4), 0.035 (Lane 3), 0.05 (Lane 2) and 0.1 (Lane 1) % (w/v) formaldehyde (FA), or molecular weight standards (labelled in kDa) (Lane 5) were subjected to SDS-PAGE, blotted onto nitrocellulose membranes and then screened with one of five monoclonal antibodies (mAb) specific for epitopes on the five PT subunits S1–S5, as indicated.

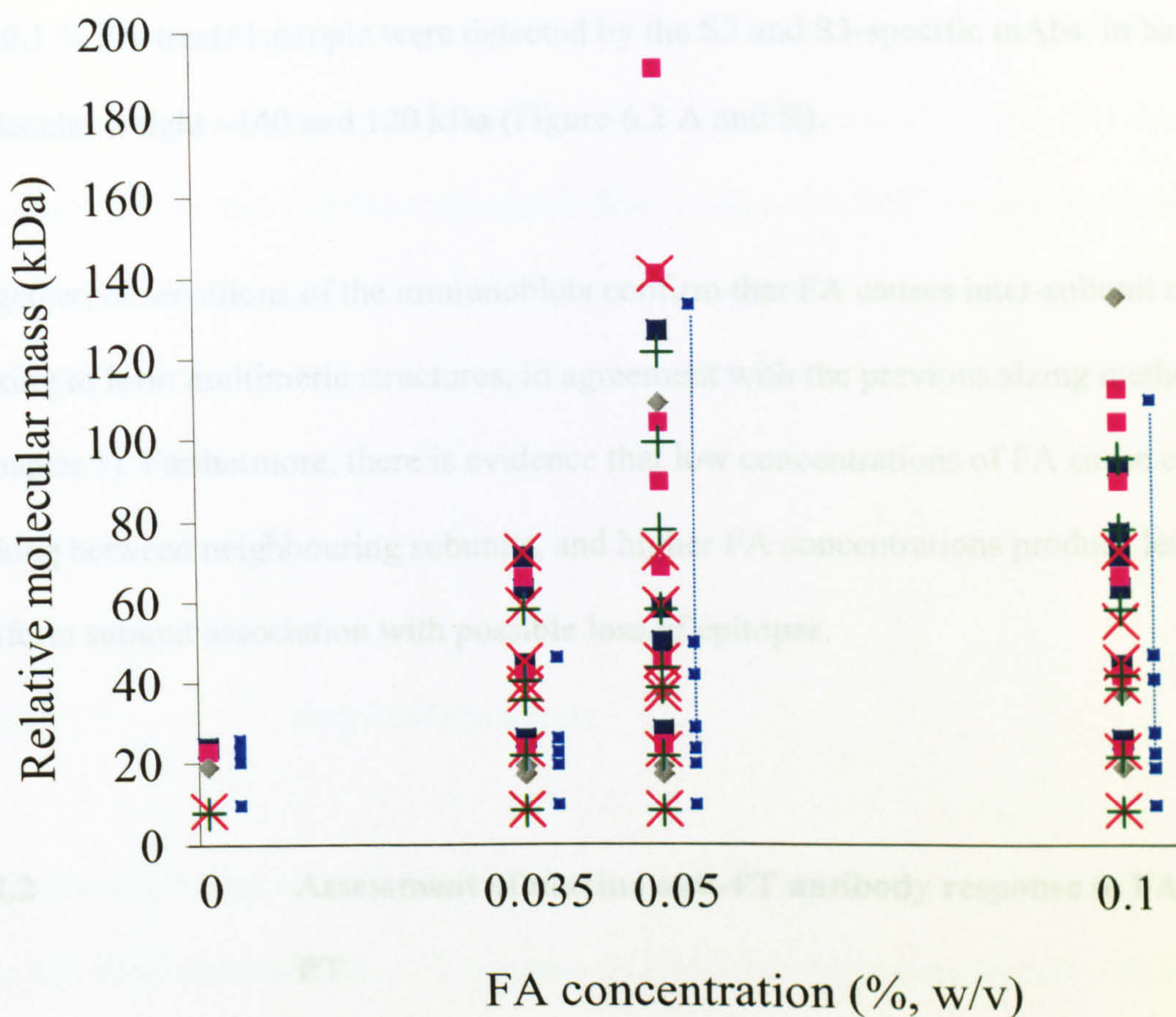


Figure 6.2 (B) PT subunit cross-linking with FA; summary

A plot of relative molecular mass as a function of % (w/v) FA for PT bands resulting from immunoblotting or Coomassie blue staining of SDS-PAGE gels. Band detected by subunit-specific mAb binding:

S1 ■ ; S2 ■ ; S3 ◆ ; S4 X and S5 +

Coomassie blue band ■ ; Coomassie blue smear: (vertical line)

the mAbs. However, fewer epitopes were detected at high molecular weights in the 0.1 % FA-treated sample compared with the 0.05 % FA-treated sample. The largest species in the 0.1 % FA-treated sample were detected by the S2 and S3-specific mAbs, in bands of molecular weight ~140 and 120 kDa (Figure 6.2 A and B).

Together, observations of the immunoblots confirm that FA causes inter-subunit cross-linking to form multimeric structures, in agreement with the previous sizing methods (Chapter 5). Furthermore, there is evidence that low concentrations of FA cause cross-linking between neighbouring subunits, and higher FA concentrations produce less uniform subunit association with possible loss of epitopes.

6.2.2 Assessment of murine anti-PT antibody response to FA-treated PT

To investigate the effect of FA on the immunogenicity of mutant and native PT, groups of five mice (strain, NIH) were immunized once, sub-cutaneously with mutant or native PT treated with 0.25 - 1.00 % FA, or untreated mutant PT; each administered with and without alum. Five weeks later, the mice were terminally bled and serum samples prepared. Two serum samples raised against 0.25 % FA-treated native PT and one against 0.5 % FA-treated native PT were not available for analysis because these mice died 10 days after immunization. This suggests that with low FA-treatment the native PT samples may still have residual toxicity and/or have reverted to toxicity *in vivo*.

The total IgG and IgG subclass response was investigated by assaying the PT-specific total IgG, IgG1 and IgG2a subclass antibodies in the serum samples using ELISA, and the effect on the antibody response of adsorbing the antigens to alum was also assessed. The binding specificity of these sera for different subunits of PT was also assessed, by an immunoblotting assay using a native PT reference preparation. In addition, the neutralizing capacity of these sera for PT was determined using the CHO-cell neutralization assay.

6.2.2.1 Total IgG response

To assess the PT-specific total IgG antibody response in mice immunised with different FA-treated mutant and native PT preparations, 96-well plates were coated with reference PT (P57), screened with the different serum samples or anti-PT reference serum (97/642, 1st International Reference Preparation (IRP)) followed by a secondary antibody, goat anti-mouse total IgG antibody. The geometric mean potency for each serum sample was calculated by parallel line analysis, relative to the anti-PT reference serum.

Mutant PT

Untreated mutant PT generated a lower total IgG response than all of the FA-treated mutant PT preparations ($P \leq 0.1$) (Figure 6.3). The 0.25 % FA-treated sample generated relatively high levels of total IgG in murine sera and there was a slight decreasing trend

in this response with increasing FA treatment. IgG levels decreased by 22 %, from a relative geometric mean titre of 1.52 for the 0.25 % FA-treated mutant PT to 1.19 for the 1.0 % FA-treated mutant PT, although this was not statistically significant ($P > 0.1$).

These results demonstrate that FA-treatment of mutant PT does not cause a significant decrease in the total IgG production compared with the untreated mutant PT, and in fact may cause a slight increase in the total IgG response. This may be due to FA-induced cross-linking causing stabilising of the antigen, or to effective presentation of epitopes to the immune system on the high molecular weight complexes.

Native PT

Untreated native PT could not be used to immunise mice for the production of antibodies due to its toxicity. Native PT treated with 0.25 % FA generated a very low total IgG response which increased dramatically with FA treatment (Figure 6.3). In response to the 0.25 % FA-treated native PT, the relative geometric mean titre for total IgG was 0.57, which is much lower than that of the corresponding mutant PT ($P \leq 0.1$). With 0.7 % FA-treated native PT the total IgG antibody increased significantly by 89 % to 5.21 ($P \leq 0.1$). With 1.0 % FA-treated native PT the antibody level dropped off slightly compared with the 0.7 % FA-treated, but this was not statistically significant. Both the 0.7 and the 1.0 % FA-treated native PT preparations induced total IgG responses which were significantly higher than the total IgG levels induced by the corresponding mutant PT preparations ($P \leq 0.1$).

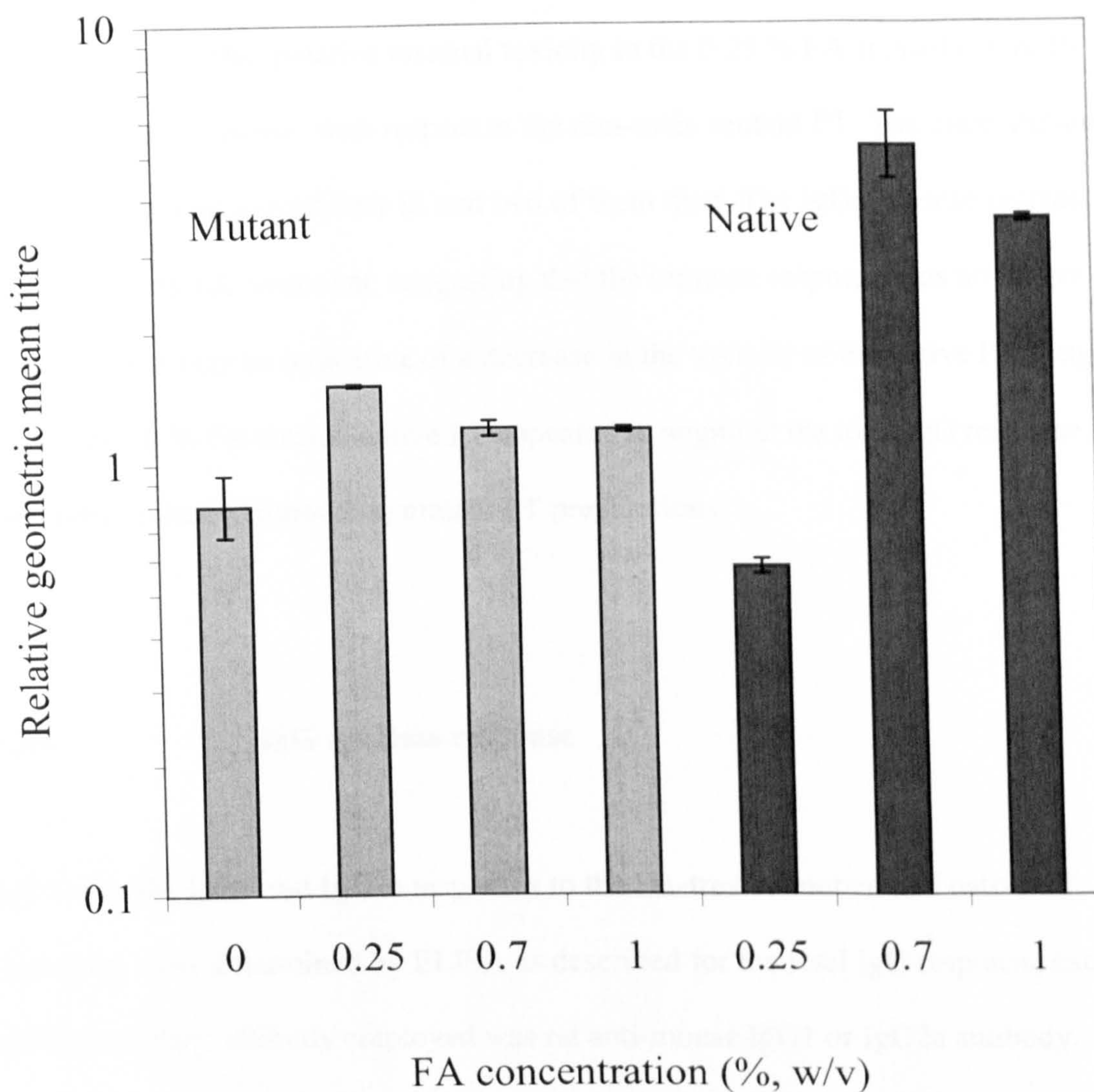


Figure 6.3 The effect of FA on anti-PT total IgG production

Serum was collected from groups of five mice each immunised with 5 μ g of mutant or native pertussis toxin (PT) treated with 0.25, 0.70 or 1.00 % (w/v) formaldehyde (FA); or untreated mutant PT. PT-specific total IgG was quantitated by ELISA relative to a PT reference serum, and expressed as the relative geometric mean of the five serum samples with one geometric standard deviation, indicated by error bars. These results are representative of three separate immunogenicity experiments.

These results imply that putative residual toxicity in the 0.25 % FA-treated native PT inhibited the IgG response, with respect to the non-toxic mutant PT. The mice immunized with this preparation looked very ill and two of them died. The IgG response increased dramatically with FA-treatment, suggesting that the immune response was no longer inhibited, which may be indicative of a decrease in the toxicity of the native PT antigen. The 0.7 and 1.0 % FA-treated native PT appeared to augment the total IgG response in comparison to the corresponding mutant PT preparations.

6.2.2.2 IgG subclass response

The PT-specific IgG1 and IgG2a responses to the FA-treated mutant and native PT preparations were determined by ELISA as described for the total IgG response, except that the secondary antibody employed was rat anti-mouse IgG1 or IgG2a antibody.

Mutant PT

The trend in the IgG1 and IgG2a response (Figure 6.4) was very similar to that of the total IgG response (Figure 6.3) with respect to FA. With 0.25 % FA-treatment of the antigen both the IgG1 and the IgG2a response increased, although this was only statistically significant for the IgG1 response ($P \leq 0.1$) (Figure 6.4). Compared with 0.25 % FA treated mutant PT, the 0.7 % FA-treated preparation induced a much lower IgG1 and IgG2a response ($P \leq 0.1$) (Figure 6.4). With further FA treatment (1.0 % FA) there was no further decrease in IgG1 and IgG2a (Figure 6.4).

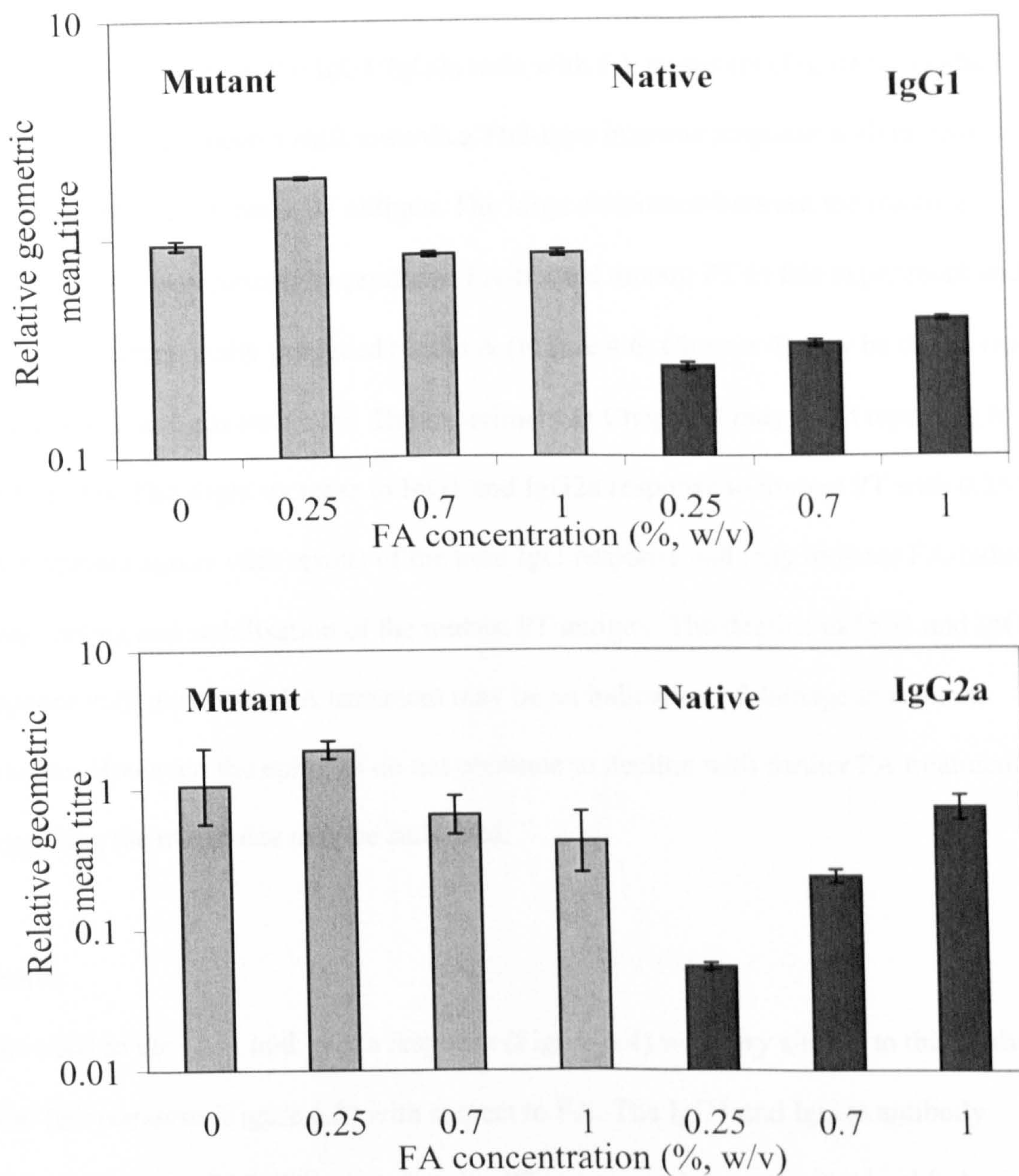


Figure 6.4 The effect of FA on anti-PT IgG subclass response

Serum was collected from groups of five mice each immunised with 5 μ g of mutant or native pertussis toxin (PT) treated with 0.25, 0.70 or 1.00 % (w/v) formaldehyde (FA). PT-specific IgG1 and IgG2a was quantitated by ELISA relative to an anti-PT reference serum, and expressed as the relative geometric mean of the five serum samples with one geometric standard deviation, indicated by error bars. These results are representative of three separate immunogenicity experiments.

The increase observed in the IgG1/IgG2a ratio with FA treatment (Figure 6.5) indicates that there may have been a shift towards a Th2-type immune response with increasing FA-treatment of the mutant PT antigen. The large difference between the immune response to the experimentally produced FA-treated mutant PT in this experiment and the analogous commercially produced toxoid A (Figure 4.6, Chapter 4) may be due to real differences in antigen treatment. The experiment in Chapter 4 may merit repeating to confirm this. The slight increase in IgG1 and IgG2a response to mutant PT with 0.25 % FA-treatment agrees with results of the total IgG response and may indicate FA-induced cross-linking and stabilisation of the mutant PT antigen. The decline in IgG1 and IgG2a response with increasing FA treatment may be an indication of damage to immune epitopes. However, the epitopes do not continue to decline with further FA treatment suggesting the molecules may be stabilised.

Native

The trend in the IgG1 and IgG2a response (Figure 6.4) was very similar to that of the total IgG response (Figure 6.3) with respect to FA. The IgG1 and IgG2a antibody responses to the 0.25 % FA-treated native PT were very low, and considerably lower than the responses to the corresponding mutant PT ($P \leq 0.1$) (Figure 6.4). From 0.25 % FA-treatment to 1.0 % FA-treatment, the IgG2a potency increased by 93 % ($P \leq 0.1$) to a level comparable with that induced by the corresponding mutant PT preparation (Figure 6.4). However, the IgG1 response to native PT did not significantly increase with FA treatment, and with 1.0 % FA-treatment was only 50 % of that induced by the corresponding mutant PT preparation.

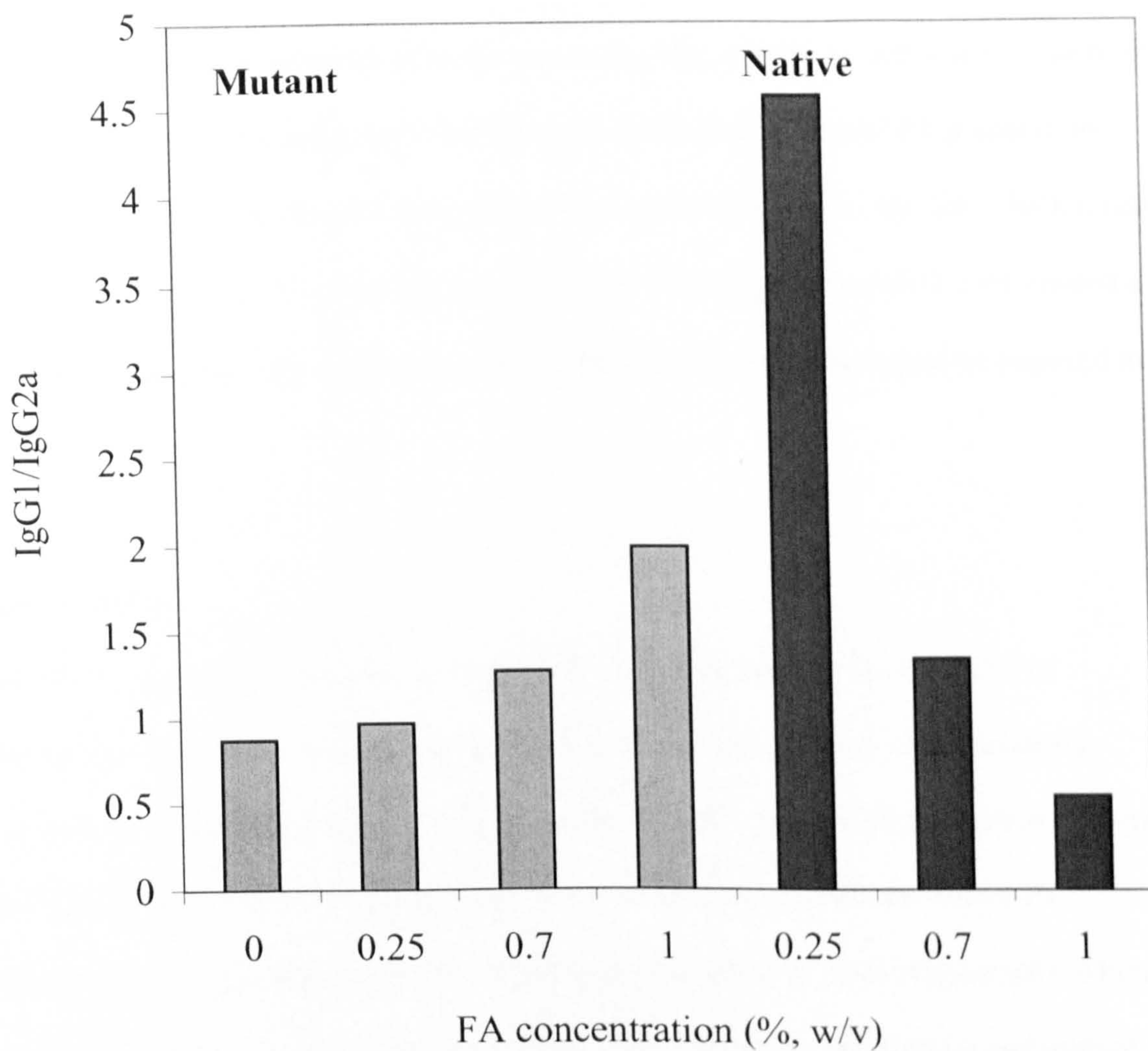


Figure 6.5 The effect of FA on the IgG1/IgG2a ratio

Serum was collected from groups of five mice each immunised with 5 µg of mutant or native pertussis toxin (PT) treated with 0.25, 0.70 or 1.00 % (w/v) formaldehyde (FA). PT-specific IgG1 and IgG2a were quantitated by ELISA relative to an anti-PT reference serum, and the geometric mean IgG1 and IgG2a titres of the five serum samples for each group were expressed a ratio of IgG1/IgG2a.

The 0.25 % FA-treated preparation induced a strong Th2 profile, as indicated by the high IgG1/IgG2a ratio; five-fold greater than for the corresponding mutant PT preparation (Figure 6.5). With increasing FA treatment of the native PT antigen, the IgG1/IgG2a ratio decreased dramatically (Figure 6.5), suggesting that FA-treatment of native PT caused a polarization towards a Th1-type response. Further cytokine studies would be required to confirm this.

Effect of alum

Most acellular pertussis vaccines are adsorbed to aluminium hydroxide (alum) or aluminium phosphate for immunisation. To investigate the effect of alum, antibody responses were also determined for mutant and native PT preparations adsorbed to alum. Total IgG data is presented for 0.25 and 1.00 % FA-treated mutant and native PT preparations both administered with and without adsorption to alum (Figure 6.6). Alum caused a significant increase in total IgG response to the FA-treated native and mutant PT preparations ($P \leq 0.1$), with the exception of the response to 1% FA-treated native PT, which was not significantly increased ($P > 0.1$). This may have been due to the fact that the IgG antibody response was already close to its upper limit. The effect of alum on the response to the 0.25 % native PT was not as great as the effect on the response to the corresponding mutant PT, which may be another result of the stress and immune suppression experienced by this group of mice, as previously discussed. The overall trend in total IgG antibody response with increasing FA-treatment was the same for the samples administered with alum (data not shown) as for those without alum (Figure 6.3). The trend in IgG1 and IgG2a with increasing FA-treatment was also similar with (data

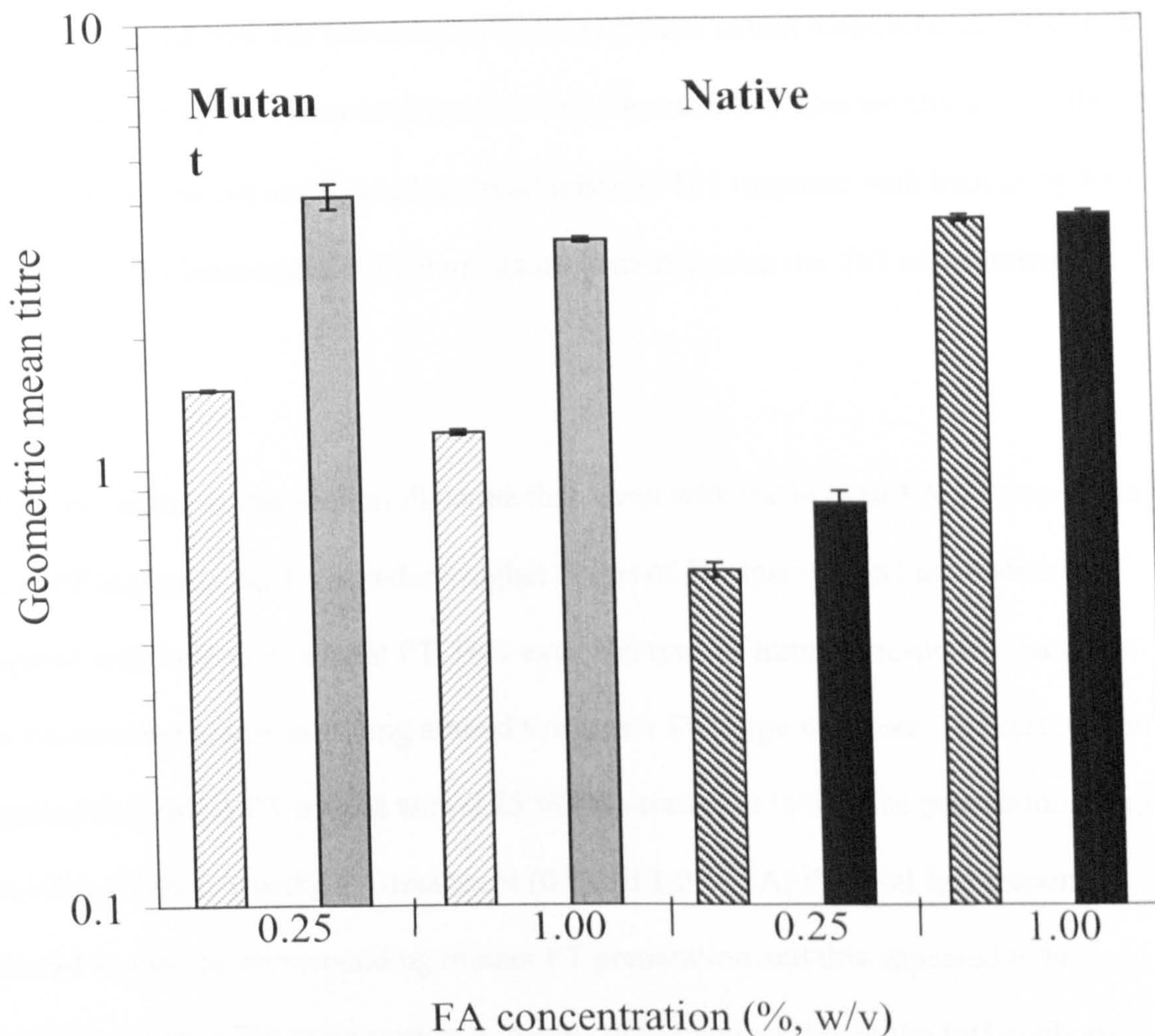


Figure 6.6 The effect of alum on total IgG production when administered with native or mutant PT

Serum was collected from groups of five mice each immunised with 5 μg of mutant or native pertussis toxin (PT), treated with 0.25 or 1.00 % (w/v) formaldehyde FA, with (block bars) or without (hatched bars) alum. PT-specific total IgG was quantitated by ELISA relative to a PT reference serum, and expressed as the geometric mean of the five serum samples with one geometric standard deviation, indicated by error bars. These results are representative of three separate immunogenicity experiments.

not shown) and without alum (Figure 6.4). Exceptionally, for mutant PT the IgG2a response decreased with FA treatment to an even greater extent when administered with alum, demonstrating the effect of alum as a Th2 adjuvant. It is also worthy to note that for native PT there was not such a marked trend towards Th1 response with increasing FA-treatment when administered with alum, again demonstrating the Th2 adjuvanticity of alum.

Overall the results in this section illustrate that, even with the highest FA treatment, the mutant PT antigens could still induce higher levels of PT-specific IgG antibodies compared with untreated mutant PT. However, the type of immune response changed with FA treatment, demonstrating a trend towards a Th2-type response. The results also suggested that native PT treated with 0.25 % FA seemed to inhibit the production of IgG antibodies. With increasing FA-treatment (0.7 and 1.0 % FA) the total IgG response exceeded that of the corresponding mutant PT preparation and this appeared to be polarized towards a Th1-type response. Alum caused an increase in the IgG antibody production but did not effect the overall FA-induced changes on mutant and native PT immunogenicity.

6.2.2.3 Binding specificity of antisera for PT subunits in immunoblots

To assess the binding specificity of the antisera for PT subunits, identical preparations of reference PT (untreated mutant PT) were subject to SDS-PAGE and blotted onto

nitrocellulose membranes. These were cut into strips encompassing one reference PT and one molecular weight marker, and each strip was screened with the sera raised against 0.25 – 1.00 % FA-treated mutant and native PT preparations, or with anti-PT reference serum (97/642, 1st IRP), and the results of the 0.25 and 0.70 % FA-treated preparations are presented (Figure 6.7).

Screening of PT blots with the anti-PT reference serum produced dense bands corresponding to subunits S1, S2 and S3, and a faint band corresponding to S4/S5 (Figure 6.7). Similar dense bands for S1, S2 and S3 were detected with serum from mice immunized with 0.25 – 1.0 % FA-treated mutant PT, and results from the 0.25 and 0.70 % FA-treated preparations are representative of these. Dense bands corresponding to S1, S2 and S3 also developed with serum against 0.50, 0.70 and 1.0 % FA-treated native PT, and the result for 0.7 % FA-treated native PT is presented (Figure 6.7). In contrast, serum from mice immunized with 0.25 % FA-treated native PT produced S1, S2 and S3 bands of distinctly lower intensity than the other samples (Figure 6.7). The S4/S5 band could not be detected by any of the test sera at the concentrations applied, suggesting that there was a very low density of anti-S4 and S5 antibodies in the sera, which may reflect the FA-induced damage to the epitopes on S4 and S5, as previously detected by ELISA (Figure 6.1). Alternatively, the anti-S4/S5 antibodies may have been abundant, but the immune epitopes may have been conformational epitopes susceptible to denaturation during SDS-PAGE; in which case the anti-S4 or S5 antibodies could not be detected by this method.

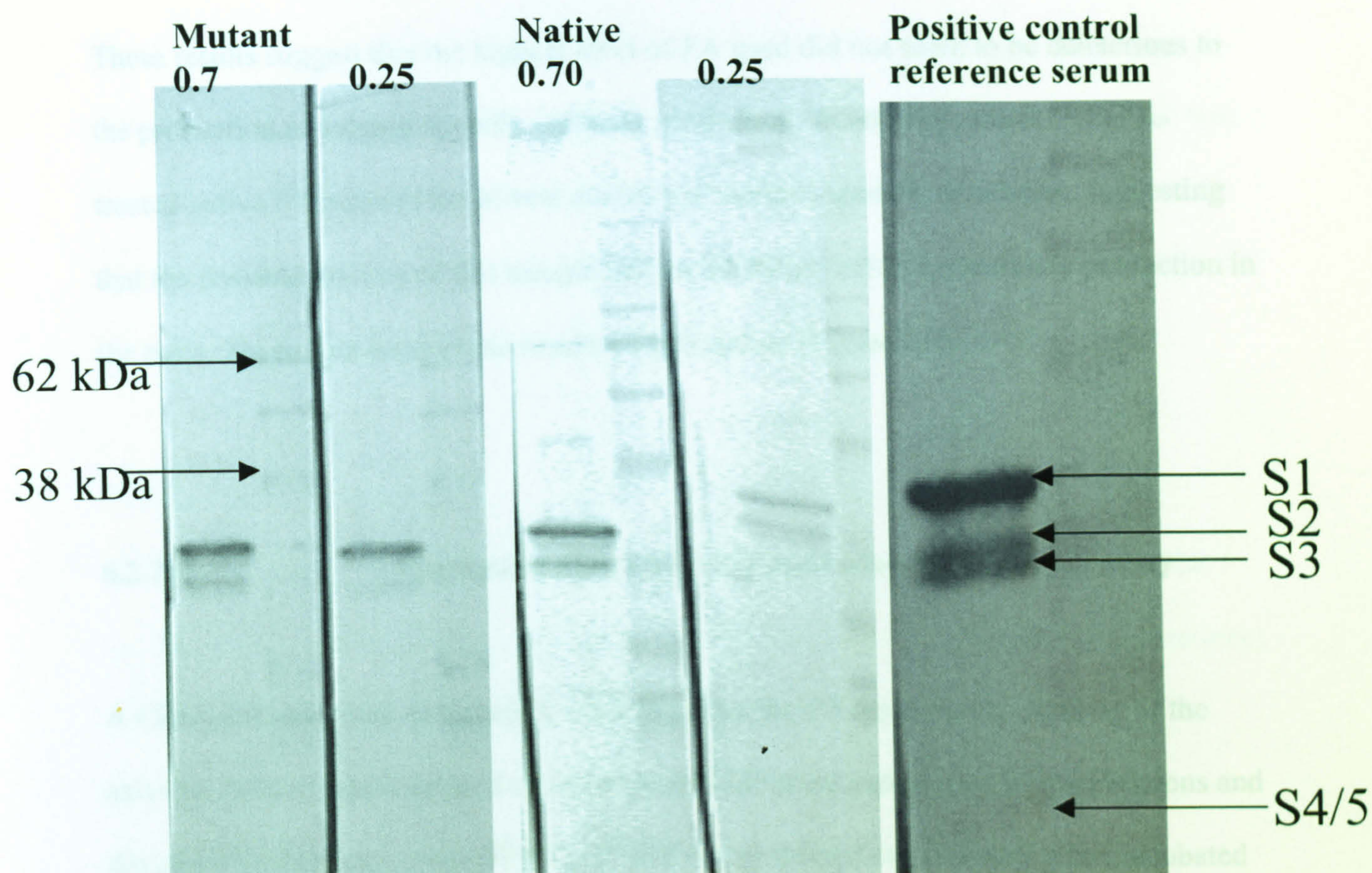


Figure 6.7 The effect of FA treatment on the quality of anti-PT serum

Serum was collected from groups of five mice each immunised with 5 μ g of mutant or native pertussis toxin (PT) treated with 0.25 or 0.70 % (w/v) formaldehyde (FA). The pooled sera from each group of five or an anti-PT reference serum were used to screen nitrocellulose membrane SDS-PAGE blots of reference PT.

These results suggest that the highest level of FA used did not seem to be deleterious to the production of subunit-specific antibody production. In addition, the 0.25 % FA-treated native PT induced the lowest quantity of subunit-specific antibodies, suggesting that the possible toxicity of this sample had an inhibitory effect on antibody production in the mice. These data support the results of IgG assays (Figure 6.3).

6.2.2.4 Assessment of neutralising antibodies by CHO-cell assay

A CHO-cell assay was designed in order to assess the PT-neutralising capacity of the antisera. Serum samples raised against FA-treated native and mutant PT preparations and the anti-PT reference serum (97/642, 1st IRP) were titrated on a 96-well plate, incubated with reference PT (90/518, NIBSC standard; 25 µL, 16 ng/mL) for three hours and then the CHO-cell suspension added and incubated for a further 2 days. The plates were assessed for neutralization of the toxic clumping effect of PT on the CHO-cells. The neutralisation titres were corrected for variation between plates relative to the reference serum titre and were expressed as the geometric mean for each group of serum samples.

Mutant

The neutralization capacity of murine serum induced by 0.25 % FA-treated mutant PT was relatively high, and this decreased with increasing FA treatment of the mutant PT antigen, declining by 60 % ($P \leq 0.1$) with 1.0 % FA (Figure 6.8).

Native

The neutralising capacity of murine serum induced by 0.25 % FA-treated native PT was much lower than that for the corresponding mutant PT preparation. In direct contrast to mutant PT results, the neutralizing capacity increased with FA-treatment of the native PT antigen, and had increased by 80 % ($P \leq 0.1$) with 1.0 % FA (Figure 6.8).

Comparison with total IgG

The trend in the neutralizing capacity of the sera with increasing FA-treatment of the antigen, mirror the trend in the IgG antibody response. From 0.25 – 1.00 % FA-treatment of the native PT antigen, there is an 84 % increase in total IgG potency and an 80 % increase in the neutralization capacity. Therefore, the increased population of IgG antibodies consisted of neutralising antibodies. With 0.25 – 1.00 % FA-treatment of the mutant PT antigen, there is a 21 % decrease in the total IgG titre but a much larger decrease of 60 % in the neutralizing capacity. This suggests a large decline in the neutralizing population of IgG antibodies, with 1.00 % FA-treated mutant PT antigen.

6.3**Discussion****Characterisation of antigens**

Immunoblotting results showed that with low FA-treatment (0.035 %) of mutant PT, cross-linking occurred between neighbouring subunits in the holotoxin structure. With higher FA-treatment, above 0.035 % FA, molecules exceeding holotoxin molecular

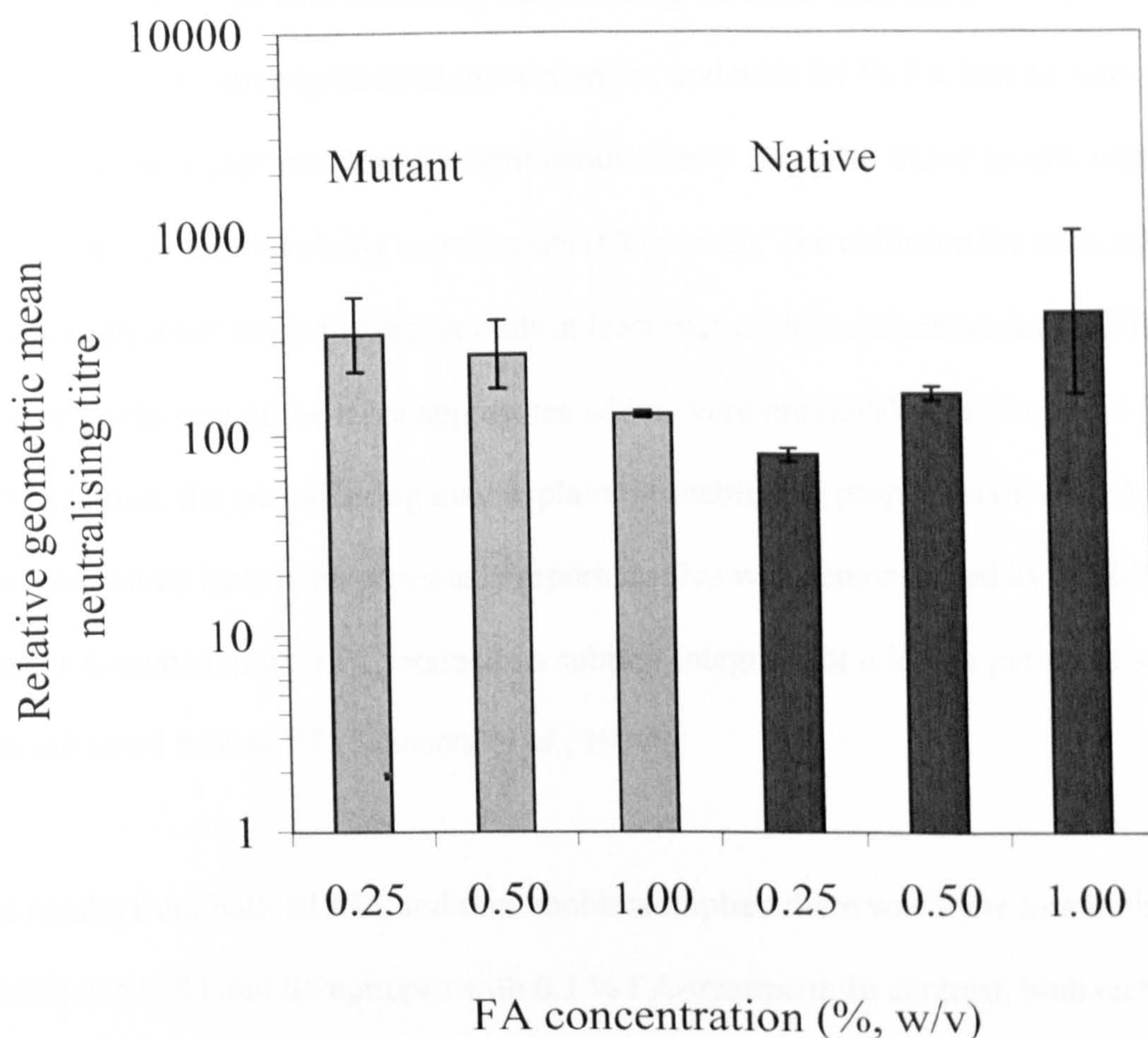


Figure 6.8 The effect of FA on anti-PT neutralising capacity

Serum was collected from groups of three mice each immunised with 5 μ g of mutant or native pertussis toxin (PT) treated with 0.25, 0.50 or 1.00 % (w/v) formaldehyde (FA). Each serum sample was titrated, incubated with active PT reference and further incubated with CHO-cells. The neutralisation titre was taken as the reciprocal of the highest serum dilution causing complete neutralisation of CHO-cell clumping. Results are expressed as the geometric mean of three serum samples and geometric standard deviation is denoted by error bars.

weight were produced demonstrating cross-linking between multimeric species. In addition, the cross-linking became more random, and with 0.1 % FA loss of epitopes was observed in the higher molecular weight bands over ~ 100 kDa. These results support those from the previous sizing experiments (Chapter 5). The evidence for inter-subunit and inter-multimer cross-linking, reveals at least part of the mechanism for the FA-induced production of the large aggregates which were previously observed (see Chapter 5). In addition, the cross-linking may explain the stabilising properties of low FA-treatment which have been previously reported. This was demonstrated by SDS-PAGE, in that FA-treated mutant PT retained its subunit integrity for a longer period of storage than untreated mutant PT (Nencioni *et al.*, 1990).

The results from both ELISA and immunoblots implied there was some loss in the density of S1, S4 and S5 epitopes with 0.1 % FA-treatment. In contrast, both techniques demonstrated that abundant S2 and S3 epitopes prevailed in this preparation. Although some correlations can be made between the results of ELISA and immunoblotting, the same significant reduction in S1, S4 and S5 subunit epitopes measured by ELISA was not observed by immunoblotting. The immunoblot bands for all subunits remained dense, even with the highest FA-treatment. This could be an indication that there was masking of the S1, S4 and S5 epitopes in ELISA, and unmasking with the denaturing technique of immunoblotting, presumably as the result of reversion of FA-induced modifications. In addition, the pattern of mAb-binding in immunoblotting confirmed that FA caused the formation of inter-subunit covalent cross-links and perhaps the cross-linking of Lys from the reaction mixture.

These results provide evidence for two possible mechanisms of epitope loss. One mechanism is by specific FA-induced modification of amino acids that comprise the subunit epitopes, and the other is by masking of intact epitopes within aggregated molecules. Chemical cross-linking between Lys from the reaction mixture and specific epitope amino acids, or intra-molecular cross-linking, could be responsible for direct epitope modification. Additionally, association of molecular surfaces by covalent cross-linking or non-covalent interactions could be responsible for epitope masking.

As previously described (section 5.3, Chapter 5), certain amino acids form reactive intermediates during reaction with FA, and these can form methylene cross-links in proteins. Lys in solution can also cross-link with protein amino acids (Tome and Naulet, 1981). The 1B7 epitope on the S1 subunit is known to reside within the amino acid sequence 8 – 15 (Sato *et al.*, 1984) and this sequence harbours four primary FA-reactive amino acids (Cieplak *et al.*, 1988). For native PT these are Tyr-8, Arg-9, Tyr-10 and Arg-13, and in mutant PT Arg-9 is changed to Lys, which is known to be the most FA-reactive amino acid (Tome, Kozlowski, and Mabon, 1985). This may account for the difference in the FA-induced modification of 1B7-binding in ELISA, observed between mutant and native PT (Figure 6.1). Some FA-induced cross-links and other modifications produced by FA have been found to be reversible (described in section 5.3, Chapter 5) (Bizzini and Raynaud, 1974; Tome *et al.*, 1979; Tome, Kozlowski, and Mabon, 1985). The occurrence of reversible interactions correlates with the mechanism of epitope unmasking. In the immunoblotting experiment it is likely that reversion of some

interactions led to unfolding or dissociation of large multimers, allowing mAbs to bind. Similar evidence of B-cell epitope unmasking *in vitro* has previously been presented (Nencioni *et al.*, 1991) and specific T-cell epitopes have also been shown to be restored after heat denaturation (Di Tommaso *et al.*, 1994).

In contrast to the other subunit epitopes, ELISA and immunoblotting experiments showed that the S2/3 epitope was not significantly altered. This may be due to low modification of this particular S2/3 epitope due to its amino acid sequence, and/or it may be that the these subunits are presented on the surface of the cross-linked molecules.

Immune response

Mutant and native PT preparations, treated with increasing concentrations of FA, produced totally different trends in antibody response, even though the corresponding mutant and native PT preparations were found to be physico-chemically similar (Chapter 5). In the characterisation of FA-induced changes in PT immunogenicity, it seemed that the most influential factor controlling the immune response to the FA-treated native PT preparations may have been their putative residual active PT. The mutant PT studies addressed the influence of structural changes alone.

The IgG response induced by 0.25 % FA-treated mutant PT preparations was higher than for the untreated mutant PT. This may be attributed to the stabilising effects of FA due to covalent cross-linking within or between mutant PT molecules as discussed above. Increasing FA-treatment did not cause the IgG titres to decline significantly, as also

observed by Nencioni and co-workers (Nencioni *et al.*, 1991). This was despite the fact that FA-treated mutant PT preparations were extensively cross-linked and aggregated with a reduced number of epitopes (section 6.2.1 and Chapter 5). These results suggest that although there was some damage to epitopes, the epitopes that were intact may be efficiently presented on aggregates to the immune system *in vivo*. It is also conceivable that the FA-aggregated structures became partly unfolded or dissociated with incubation in the body, providing a slow release of immune epitopes.

The type of antibody response induced by mutant PT may have changed with increasing FA-treatment however. This is demonstrated in that the neutralising capacity of the anti-PT antibodies declined to a greater extent than the total IgG. This suggests that FA affected the quality of the antibody response induced. This is in line with the proposal that there are local modifications to the surface protective epitopes by FA-induced cross-linking, as shown with mAb binding in ELISA and immunoblots. In addition, the relative potencies of IgG1 and IgG2a subclasses suggest that the class of immune response is shifted towards a Th2-type response. An explanation for this shift may be due to FA-induced cross-linking which could potentially alter binding to and/or processing by antigen presenting cells, thus altering the cytokine environment. This explanation is in line with evidence, from *in vitro* studies of T-cell proliferation, that FA-treatment of vaccine antigens alters their proteolytic processing by antigen presenting cells (Di Tommaso *et al.*, 1994).

Antibody production induced by the 0.25 % FA-treated native PT was significantly lower than the response induced by the corresponding mutant PT preparation. The 0.25 % FA-treated native PT was potentially the most toxic of the native PT preparations, and concurrent with this there were in fact two fatalities in this group of mice and the remaining mice appeared stressed. Therefore, it may be that the potentially higher toxicity in this preparation was inhibitive to the immune response. With 0.7 and 1.0 % FA-treatment of native PT, a huge increase in the IgG antibody response was observed, suggesting that the antibody response was no longer inhibited by the toxicity of these preparations. These results provide some evidence that a FA concentration greater than 0.25 %, under the current conditions of preparation, is required to detoxify native PT.

It is particularly interesting to note that the total IgG response for the 0.70 and 1.00 % FA-treated native PT preparations was even higher than that for the corresponding mutant PT preparations. This implies that these FA-treated native PT preparations actually enhanced the IgG response. Although it has been suggested that the putatively high toxicity of the 0.25 % FA-treated PT inhibited the total IgG response, it may be that much lower residual activity can have the opposite effect.

Concomitant with the augmented total IgG levels, the quality of the antibody response was altered. The differential induction of IgG1 and IgG2a suggests that the enhanced response was Th1 polarised. In addition, the large number of antibodies was also found to consist mainly of effective neutralising antibodies (Figure 6.8).

In line with the augmented antibody responses observed, native PT has been shown to have adjuvant properties. PT coadministered with immunologically inert antigens can augment murine antibodies and cytokines specific for that antigen (Ryan *et al.*, 1998b; Wilson *et al.*, 1993; Shive *et al.*, 2000). Additionally a residual amount of native PT can induce enhanced protection against *B. pertussis* infection in mice when co-administered with other *B. pertussis* antigens and cause a corresponding increase in the nitric oxide production of macrophages (Robinson and Irons, 1983; Xing, Canthaboo, and Corbel, 2000). These factors can be indicative of a Th1-polarised response, which is concurrent with the augmented immune response found in this study. Specific cytokine studies demonstrated that the adjuvant response was a mixed Th1/Th2 response (Ryan *et al.*, 1998b). There is still controversy over the mechanism of PT adjuvanticity. The results of this study, which have provided a parallel comparison of structurally similar non-toxic and partly toxic native PT preparations, suggest that the immune enhancing properties of PT are dominated by the ADP-ribosyl transferase-dependent effects.

It is interesting to note that the structurally related toxins, cholera toxin (CT) of *Vibrio cholerae* and heat-labile enterotoxin (LT) of *Escherichia coli*, also have potent adjuvant activity (Williams, Hirst, and Nashar, 1999). However, CT augments predominantly a Th2-type immune response (Wilson *et al.*, 1993), and LT a mixed Th1/Th2 response (Takahashi *et al.*, 1996). Differences in the immunogenicity of CT, LT and PT could be due to their different host cell receptors, and in the case of PT, different host G-protein substrate. The current experimental evidence suggests roles for both the A- and the B-

domains of CT and LT in their adjuvant activity (Williams, Hirst, and Nashar, 1999; Rappuoli *et al.*, 1999).

Overall it appears that FA-induced structural changes can influence epitope presentation, the inhibition or enhancement of the immune response, the neutralising quality of the antibody response and the differential induction of Th1 and Th2 cells. Variations in PT antigens such as these may help to explain the large differences in immunogenic properties between different acellular pertussis vaccines (Ausiello *et al.*, 1997; Mills *et al.*, 1998; Ryan *et al.*, 1998a). This work provides the basis for tailoring a protective immune response against *B. pertussis* by the inclusion of different quantities of molecular aggregates/cross-linked species. In addition, it suggests that ADP-ribosylating activity may affect the magnitude and class of immune response induced by PT. To corroborate the role of toxicity in the immune responses obtained in this chapter, the toxic biological activities of the native PT preparations are assessed in the next chapter (Chapter 7).

7.1**Introduction**

PT has been characterized over the decades under many different aliases, owing to its diverse and numerous biological activities. Some of these activities include its ability to sensitise animals to histamine, promote elevated levels of circulating lymphocytes, enhance insulin release, augment the immune response to coinjected antigen and, amongst its abundant *in vitro* effects, cause CHO-cells growing in a uniform mono-layer to morph and clump together (Munoz, 1985).

PT is of the AB class of protein toxins, which encompass two functionally distinct domains. The B-oligomer facilitates host-cell binding and entry of PT into the cell, and there it exerts its toxic effects via its catalytic A-domain, which ADP-ribosylates host G-proteins. The toxic biological activities of histamine sensitization, leukocyte promoting activity and CHO-cell toxicity all require functional A and B-domains and have been found to be ultimately dependent on the catalytic activity of the A-domain, that is, they cannot be caused by B-oligomer binding alone (Nencioni *et al.*, 1991). The distinct roles of A- and B-domains in the adjuvant activity of PT is unclear.

FA is commonly used to toxoid PT, namely to remove the toxic biological activities of PT, for its inclusion in acellular pertussis vaccines. Existing studies on the effects of FA

on the toxicity of native PT were carried out over a decade ago, generally on combined antigens or crude PT preparations. The Japanese DTP vaccine containing the acellular pertussis component, which encompasses 0.4 % FA-treated PT, was reported to have one-tenth of the toxicity of the whole cell vaccine as determined by mouse-body-weight-decreasing activity, leukocyte promoting activity and histamine sensitising tests (Sato, Kimura, and Fukumi, 1984). Studies of a partially purified PT extract after treatment with 0.05 – 0.2 % FA for 24 hrs at 37°C demonstrated a reduction in CHO-cell toxicity, leukocytosis and histamine sensitisation with increasing FA treatment (Gupta *et al.*, 1991). It was noted that, even with 0.2 % FA-treatment, high residual activity of PT still remained in the preparation.

In the previous chapter (Chapter 6), the effects of increasing FA treatment on the immunogenicity of PT were assessed, and opposite trends in IgG response were observed for native and mutant PT antigens. In comparison with the corresponding mutant PT preparations, the lowest FA-treated native PT seemed to inhibit the immune response, while the highest FA-treated native PT augmented the response. It was noted that there were some fatalities after 10 days, in the groups of mice inoculated with 0.25 and 0.50 % FA-treated native PT. This suggested that these lower FA-treated native PT preparations may have had residual toxicity or have reverted to toxicity *in vivo*. Accordingly, the large differences observed in the immune response induced by the native PT preparations, compared with mutant PT preparations, were attributed to their putative toxicity.

Although native PT detoxified with FA is still being used in pertussis vaccines worldwide, no new efforts have been made to characterize the detoxification process. In particular, the specific effects of FA on the functional domains of native PT, to our knowledge, have not yet been explored. In addition, the role of toxicity in the immune responses previously observed (Chapter 6) must be addressed; therefore a detailed investigation of the toxic properties of the FA-treated native PT preparations is required. The aim of these experiments was to assess the toxic properties of the FA-treated native PT preparations and to test for reversion to toxicity.

Native PT was treated with 0.25, 0.50, 0.70 and 1.00 % FA (as used in Chapter 6), which covers the range of FA concentrations used to treat PT for use in commercial pertussis vaccines. The effect of FA on the toxic biological activities of native PT was assessed using four different assays. These were, an in vitro ADP-ribosylation assay in a cell-free system, a CHO-cell toxicity assay, and two murine in vivo tests, of leukocyte proliferation and histamine sensitisation. Reversion to toxicity was also tested by analysing the toxicity of the preparations before and after incubation for 11 days at 37 °C, using the ADP-ribosylation and CHO-cell toxicity assays.

7.2 Results

7.2.1 Effect of FA on the toxic activities of native PT

Histamine sensitisation test

Five mice per group were each immunised with 5 µg/dose of one of the 0.25 – 1.0 % FA-treated native PT preparations, 100 ng/dose of the positive control active PT (90/518, NIBSC reagent) or one of the negative controls, PBS and non-toxic mutant PT (5 µg/dose). After five days each mouse was challenged with histamine diphosphate solution and the reaction of the mice was recorded.

After histamine challenge, all of the mice inoculated with PBS or mutant PT and two out of five mice inoculated with 100 ng of reference PT survived. In contrast, none of the mice from the groups inoculated with any of the FA-treated native PT preparations survived (Table 7.1). The groups injected with 0.25 and 0.5 % FA-treated native PT died more rapidly than those injected with the 0.7 and 1.0 % FA-treated native PT (Table 7.1).

From comparison with the reference PT (100 ng/dose), the results from this assay suggest that all of the FA-treated native PT samples, contain more than 100 ng of active PT per dose. This may explain the fatalities and illness of the mice that were immunised with

Table 7.1 Effect of FA treatment of native PT on histamine sensitization
Five mice per group were immunised with 0.25 – 1.00 % (w/v) formaldehyde (FA)-treated native pertussis toxin (PT) (5 µg), positive control (100 ng reference PT, code 90/518, NIBSC reagent) or negative controls (untreated mutant PT (5 µg); or PBS). The histamine sensitivity of each group was assessed and expressed as the number of fatalities per group of five.

Immunising preparation	Formaldehyde treatment (% w/v)	Number of mice, out of five, dead after histamine sensitisation
Native PT	0.25	5 (at 10 mins)
”	0.50	5 (at 10 mins)
”	0.70	5 (at 15 mins)
”	1.00	5 (at 15 mins)
PBS	-	0
Mutant PT	0	0
100 ng reference PT	0	3

0.25 and 0.5 % FA-treated native PT, observed in the immunogenicity study in the previous chapter (Chapter 6).

Leukocyte proliferation test

Mice were immunised as described for the histamine sensitisation test. Three days after immunisation, tail bleeds were taken from each mouse and the number of leukocytes in each whole blood sample was counted.

A base-line number of leukocytes (~ 6 000) was counted in the whole blood from mice inoculated with the negative control, PBS (Figure 7.1). The same level of leukocyte proliferation was obtained in response to the mutant PT sample. There was a 1.6 times greater number of leukocytes in the mice immunised with 100 ng of the reference PT than those with the base-line number. An even greater rise in the leukocyte count to > 30 000, more than 5 times higher than the base-line number, was observed in mice inoculated with the 0.25 and 0.5 % FA-treated native PT preparations (Figure 7.1). The leukocyte proliferation declined with increasing FA treatment of the native PT and was reduced by 55 % with the highest FA-treatment.

All of the FA-treated native PT preparations induced a higher leukocyte count than 100 ng of reference PT. In agreement with the histamine sensitisation test, this suggests that they all contained over 100 ng of active PT, which may explain the fatalities and illness observed in the two groups of mice in the immunogenicity study (Chapter 6).

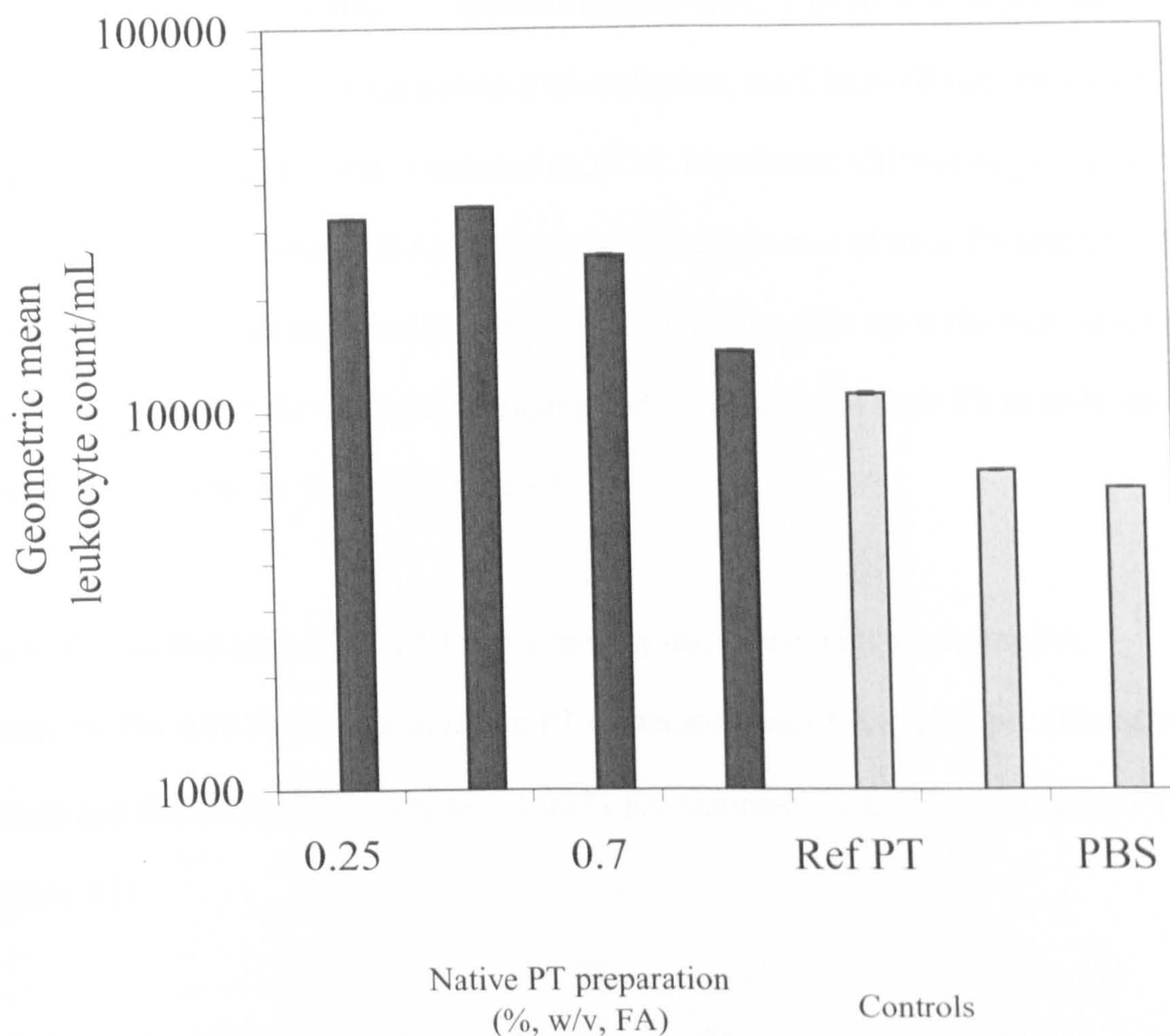


Figure 7.1 Effect of FA on leukocyte proliferation activity of native PT

Five mice per group were immunised with 0.25 – 1.00 % (w/v) formaldehyde (FA)-treated native pertussis toxin (PT) (5 µg), positive control (100 ng ref. PT, code 90/518, NIBSC reagent) or negative controls (untreated mutant PT; or PBS). The geometric mean leukocyte counts per mL of whole blood for each group of five mice were determined, and are presented with error bars indicating one standard deviation.

CHO-cell toxicity assay

Each FA-treated PT preparation, the positive control active PT (90/518, NIBSC reagent) or PBS negative control were titrated on a 96-well plate, the CHO-cell suspension was added to each well and the plate incubated at 37 °C. Unaffected CHO-cells grow in a uniform mono-layer (Figure 7.2 A), while those in the presence of toxic PT change morphology and clump together (Figure 7.2 B). After three days the wells were checked under the microscope for CHO-cell clumping and the quantity of toxic PT in each sample was determined relative to the reference PT.

The CHO-cell toxicity of native PT progressively decreased with increasing FA treatment. The 0.25 % FA-treated native PT contained 1 µg of active PT per 100 µg of protein and this declined by 95 % with 1.00 % FA treatment, to 0.053 µg of active PT (Figure 7.3).

The reduction in CHO-cell toxicity with increasing FA concentration could be as a result of alterations within the CHO-cell binding sites of the B-oligomer, which could prevent initial binding of the PT molecule to the CHO-cell. There may also or alternatively have been modifications to the amino acids of the catalytic site in the A-domain, thus inactivating the ADP-ribosyl transferase activity of the toxin. In order to address the question of the site of FA-induced detoxification, the FA-treated native PT samples were also analysed by a cell-free ADP-ribosylation assay.

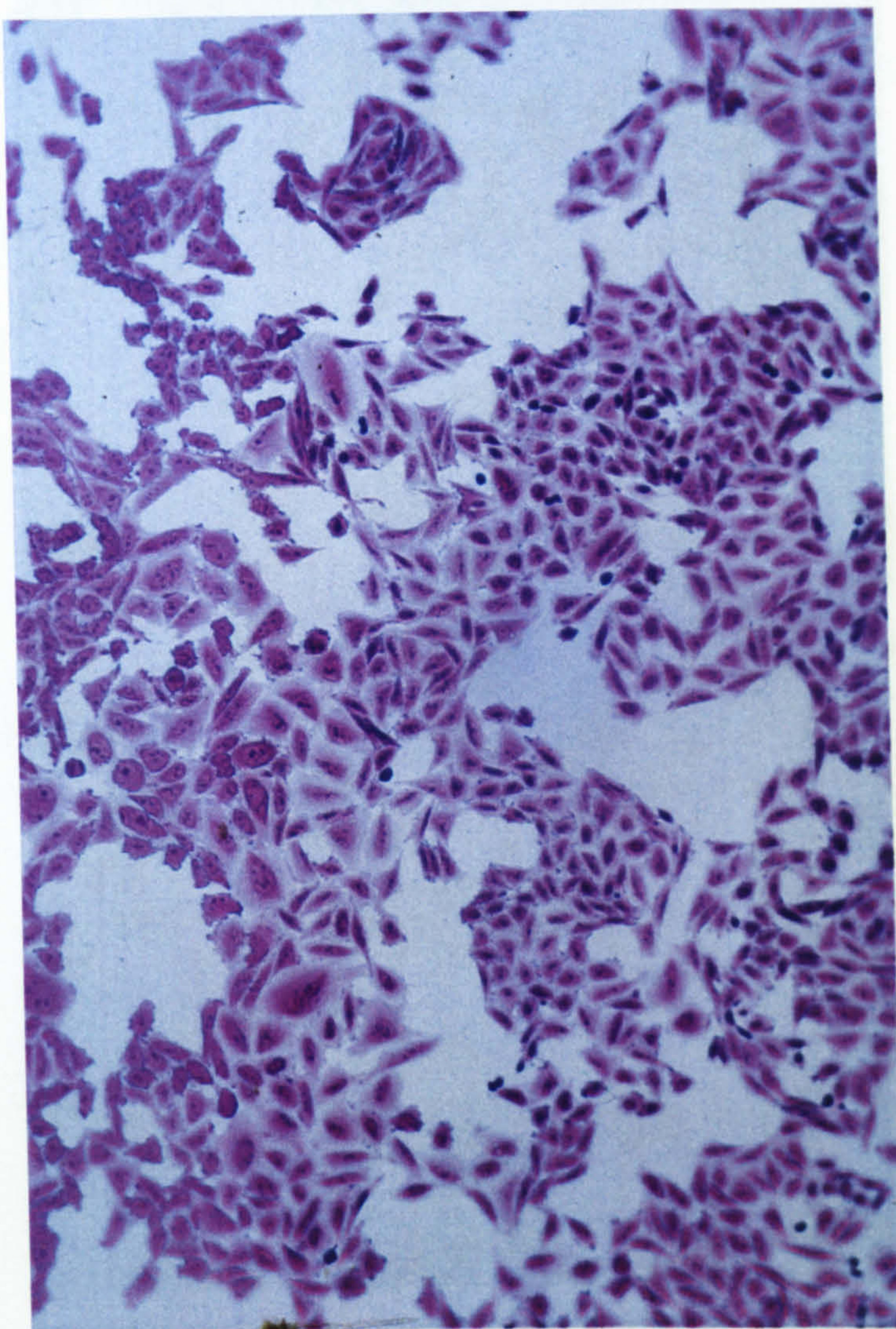


Figure 7.2 (A) CHO-cells

CHO-cells growing in a confluent monolayer. CHO-cells were grown in complete RPMI medium for 3 days at 37 °C, in 5 % CO₂. They were stained with Coomassie blue and visualised with a light microscope at 10-fold magnification. This photograph is courtesy of Dr D. Xing.

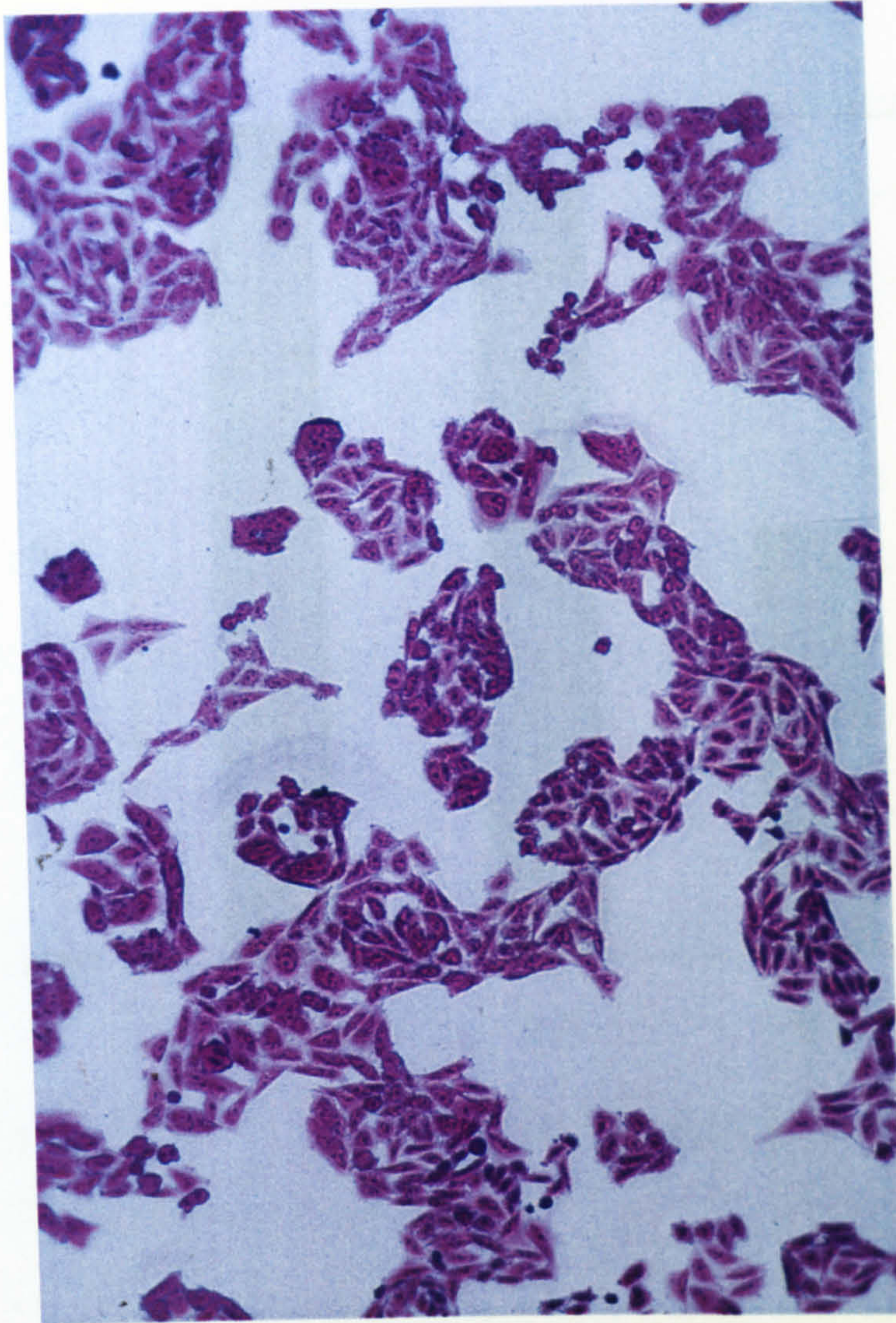


Figure 7.2 (B) CHO-cells exposed to native PT

CHO-cells were grown in complete RPMI medium, in the presence of 16 ng/mL reference PT (90/518, NIBSC reagent) for 3 days at 37 °C, in 5 % CO₂. They were stained with Coomassie blue and visualised with a light microscope at 10-fold magnification. This photograph is courtesy of Dr D. Xing.

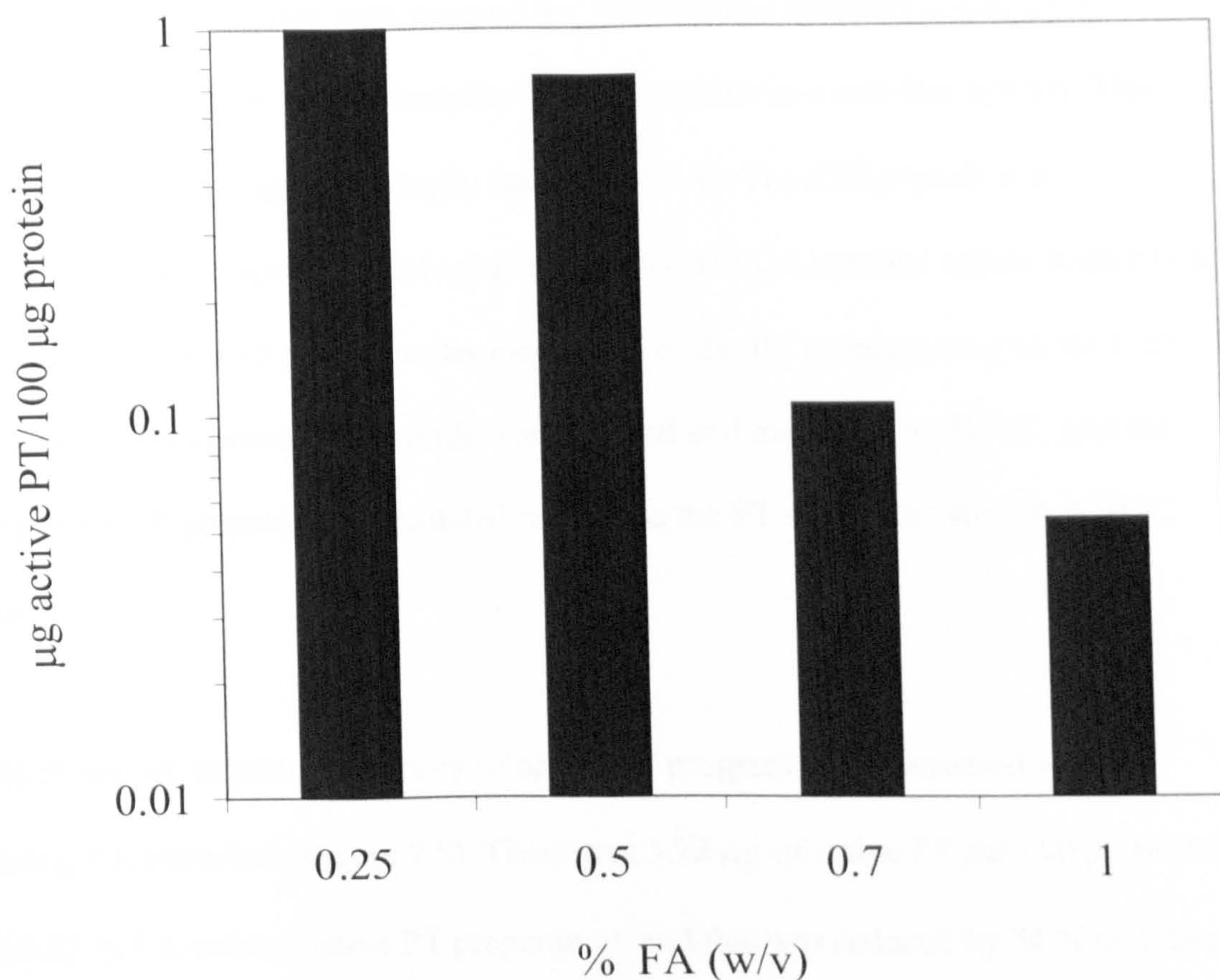


Figure 7.3 The effect of FA on CHO-cell toxicity of native PT

The quantity of pertussis toxin (PT) with CHO-cell toxicity, in native PT preparations treated with 0.25 - 1.00 % (w/v) formaldehyde (FA), was determined. The CHO-cell toxicity assay measured the ability of the preparations to cause CHO-cell clumping, performed according to Gillenius et al., 1985. The amount of active PT (μg) in each preparation was determined relative to reference PT (code 90/518, NIBSC reagent) and presented as a proportion of 100 μg of total protein in the sample. The CHO-cell toxicity assay was repeatable to one dilution factor.

ADP-ribosylation assay

The FA-treated preparations were assayed for ADP-ribosyl transferase activity by measuring their ability to ADP-ribosylate the C20 peptide in a cell-free system. This reaction is explained diagrammatically (see Figure 7.4). The C20 peptide is a fluorescently tagged peptide homologous to the 20 carboxyl-terminal amino acids of the alpha-subunit of the Gi3 protein. After incubation of the PT preparation with the C20 peptide, ADP-ribosylated C20 peptide was detected and measured by HPLC, and the activity of the PT preparation calculated relative to the PT reference (90/518, NIBSC reagent).

The ADP-ribosyl transferase activity of native PT progressively decreased with increasing FA treatment (Figure 7.5). There was 5.72 μg of active PT per 100 μg protein in the 0.25 % FA-treated native PT preparation, and this was reduced by 74 % to 1.48 μg active PT with 1.00 % FA treatment.

These results confirm that at least part of the FA-induced modification occurs within the active site of the A-domain. In order to assess whether FA-induced modifications also specifically affect the function of the B-oligomer, the results from the CHO-cell toxicity assay and ADP-ribosylation assay were directly compared.

The quantity of active PT determined by the CHO-cell toxicity assay was lower than that determined by the ADP-ribosylation assay for all of the PT preparations (Figure 7.6).

From 0.25 to 1.00 % FA-treatment, the CHO-cell toxicity decreased by 95 %, which was

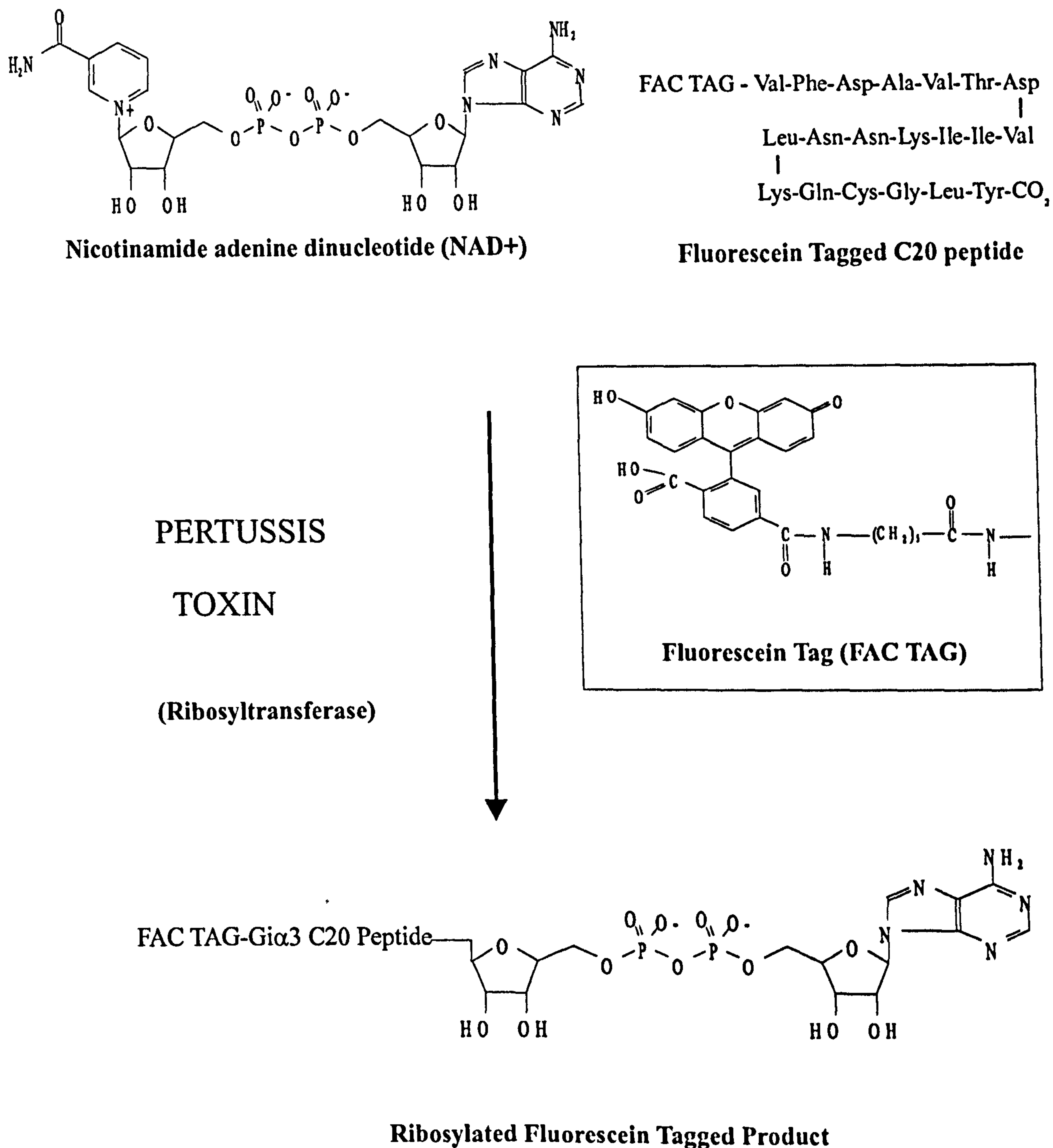


Figure 7.4 ADP-ribosylation of C20 substrate by PT

A diagrammatic representation of the pertussis toxin (PT)-catalysed ADP-ribosyl transfer from NAD⁺ to the fluorescein-tagged (FAC TAG) C20 peptide (Cyr,T.D., *et.al.*, 2001). The C20 peptide is homologous to the 20 carboxyl-terminal amino acids of the alpha-subunit of the G_{i3} protein.

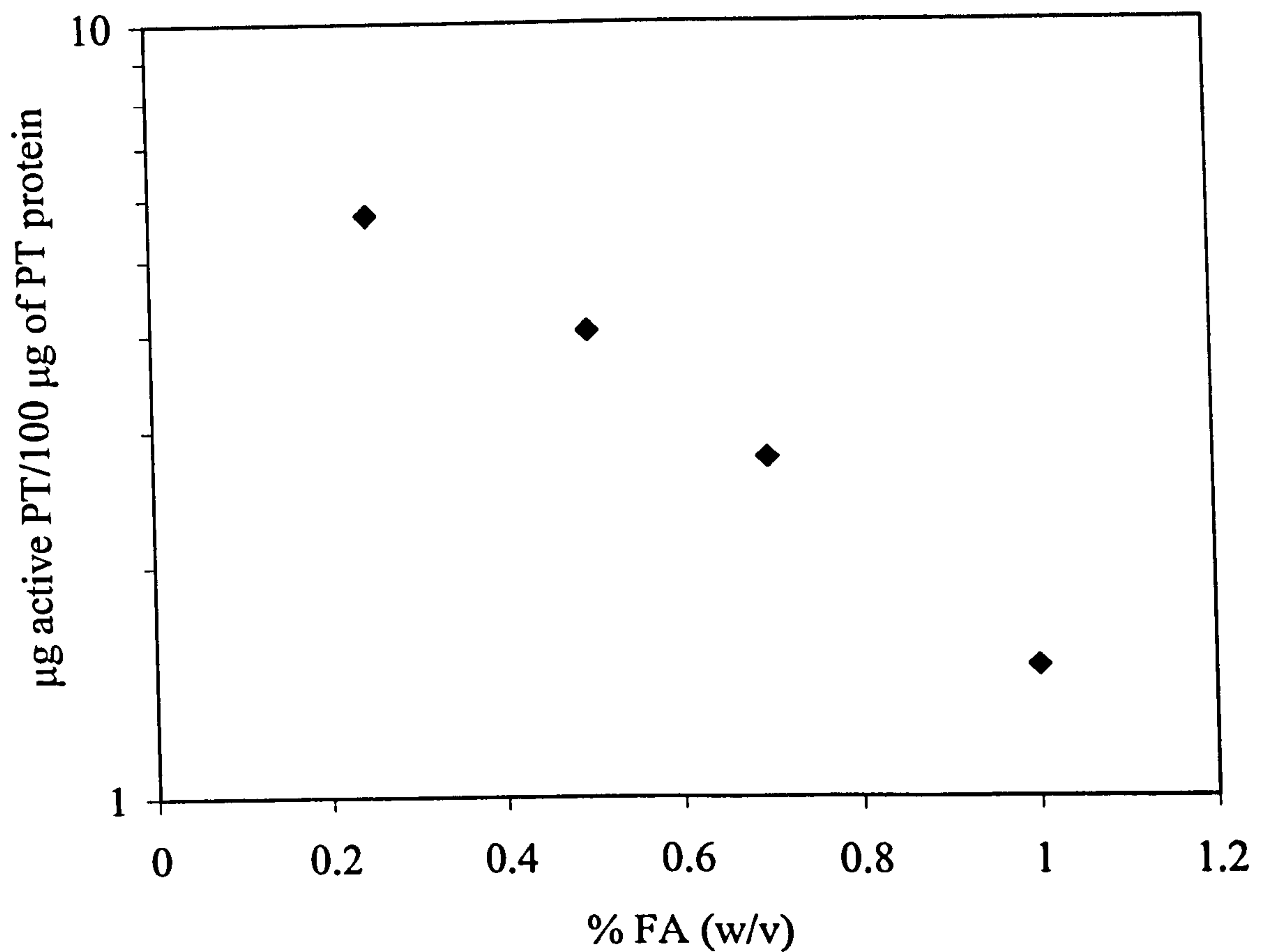


Figure 7.5 The effect of FA on ADP-ribosylation activity of native PT
The quantity of pertussis toxin (PT) with ADP-ribosyl transferase activity was determined, for native PT preparations treated with 0.25 - 1.00 % (w/v) formaldehyde (FA), . The ADP-ribosylation assay quantitated the ribosylation of a fluorescent- tagged peptide substrate using an enzymatic-HPLC coupled assay (Cyr et al., 2001). The amount of active PT (μg) in each preparation was determined relative to reference PT (code 90/518, NIBSC reagent) and presented as a proportion of 100 μg of total protein in the sample. The ADP-ribosylation assay was repeatable to ± 0.15 μg active PT/100 μg protein. These assays were performed by Dr C-T Yuen.

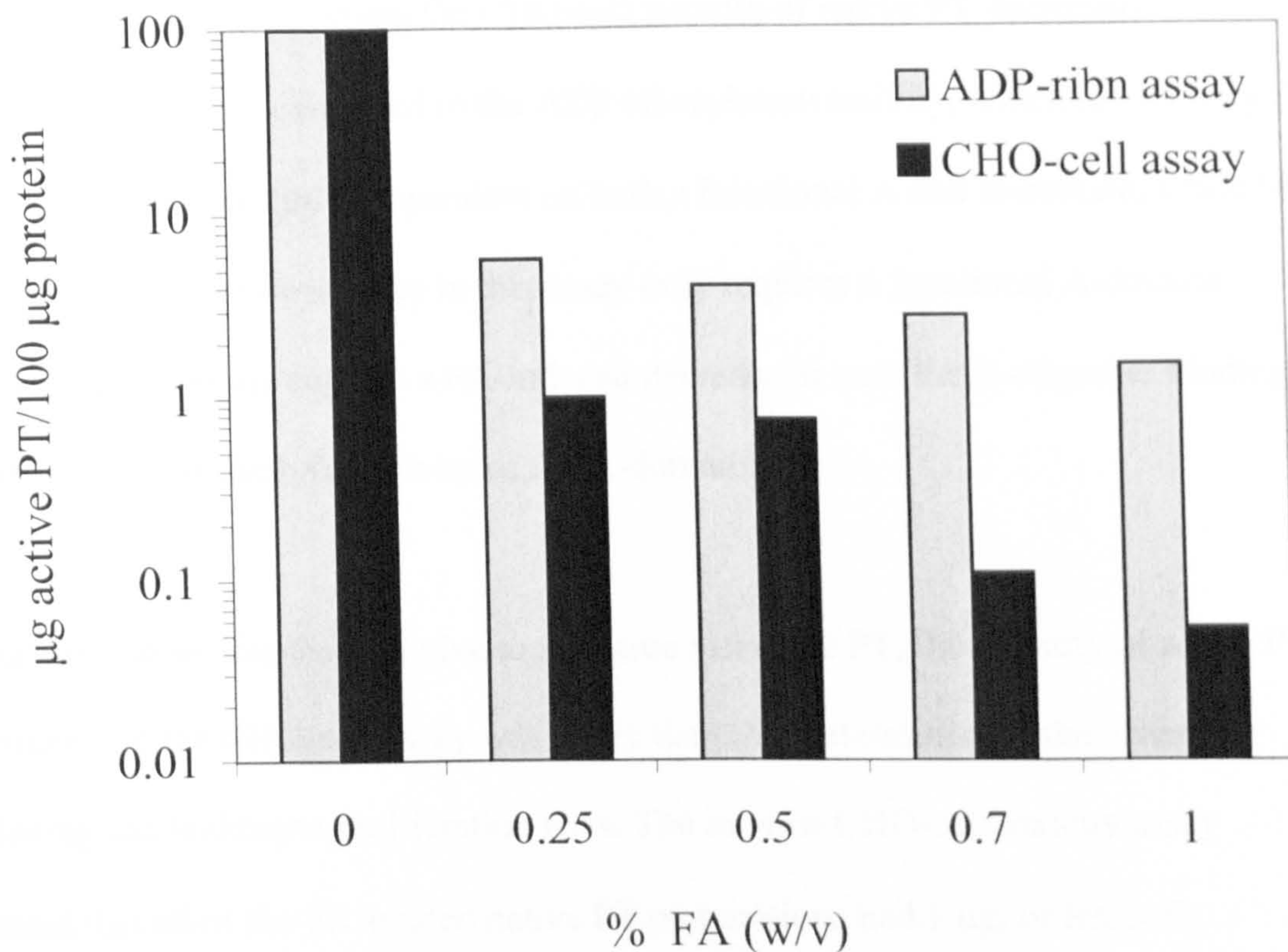


Figure 7.6 The effect of FA on ADP-ribosylation activity and CHO-cell toxicity of native PT

The quantity of pertussis toxin (PT) with CHO-cell toxicity and ADP-ribosyl transferase activity in native PT preparations treated with 0.25 - 1.00 % (w/v) formaldehyde (FA), was determined and presented as described for Figures 3 and 5 respectively. An arbitrary value of 100 μg active PT/100 μg protein has been entered for 0 % FA, for comparison.

more marked than the 74 % decline in ADP-ribosyl transferase activity. In particular, from 0.5 to 0.7 % FA-treatment the CHO-cell toxicity of native PT decreased dramatically by 86 %, compared to the ADP-ribosylation activity, which fell by only 32 %. The CHO-cell toxicity is dependent on both a functional A and B-domain, while the ADP-ribosyl transferase activity in this assay only requires a functional A-domain. Therefore these results suggest a FA-induced decrease in both the B-oligomer binding activity and in the catalytic activity of the A-domain.

It is interesting to note that, relative to the same reference PT, the quantity of active PT determined by the CHO-cell assay was lower than that determined by the histamine sensitising and leukocyte proliferation tests. The *in vitro* CHO-cell toxicity assay indicated that all of the FA-treated native PT preparations had 1 µg, or less, of biologically active PT per 100 µg of protein. However, the *in vivo* tests suggested that all of the FA-treated preparations contained much more than 2 µg per 100 µg of protein (i.e. more than 100 ng per dose: the quantity of reference PT used). A possible explanation for the discrepancy between these assays is that FA treatment may cause a higher degree of modification to the moieties on PT required for CHO-cell toxicity, than to the moieties required to produce histamine sensitisation and leukocyte proliferation *in vivo*. Another likely explanation is that the preparations reverted to toxicity *in vivo*. This has previously been demonstrated in FA-treated PT (Quentin-Millet *et al.*, 1988), and is in agreement with the observation that there were fatalities in the groups of mice immunised with 0.25 and 0.5 % FA-treated mice ten days after immunisation. A toxicity reversion study was subsequently carried out to address this.

7.2.2 Reversion to toxicity of FA-treated native PT

A quantity of each of the 0.5 and 1.0 % FA-treated native PT preparations was incubated at 37 °C for 11 days with manual shaking twice every day, and the remaining sample was left in storage at 4 °C. The incubated and the 4 °C samples were analysed by the CHO-cell toxicity assay and ADP-ribosylation assay (described in section 7.2.1).

Of the FA-treated native PT preparations tested, both the CHO-cell toxicity and the ADP-ribosyl transferase activity significantly increased after incubation at 37 °C for 11 days (Figure 7.7). The CHO-cell toxicity of the 0.5 and 1.0 % FA-treated preparation increased by 99 % and 83 % respectively, while the ADP-ribosyl transferase of these preparations increased by 33 % and 45 % respectively. These results imply that there is reversion of FA-induced detoxification. In addition, since the CHO-cell toxicity increases to a greater extent than the ADP-ribosyl transferase activity, the results suggest that there was reversal of FA-induced modifications in both the A- and B-domain.

7.3 Discussion

The results from ADP-ribosylation, CHO-cell toxicity, leukocyte proliferation and histamine sensitization tests all provided evidence that FA causes a decrease in the toxic biological activities of a purified preparation of native PT. In addition, the results show

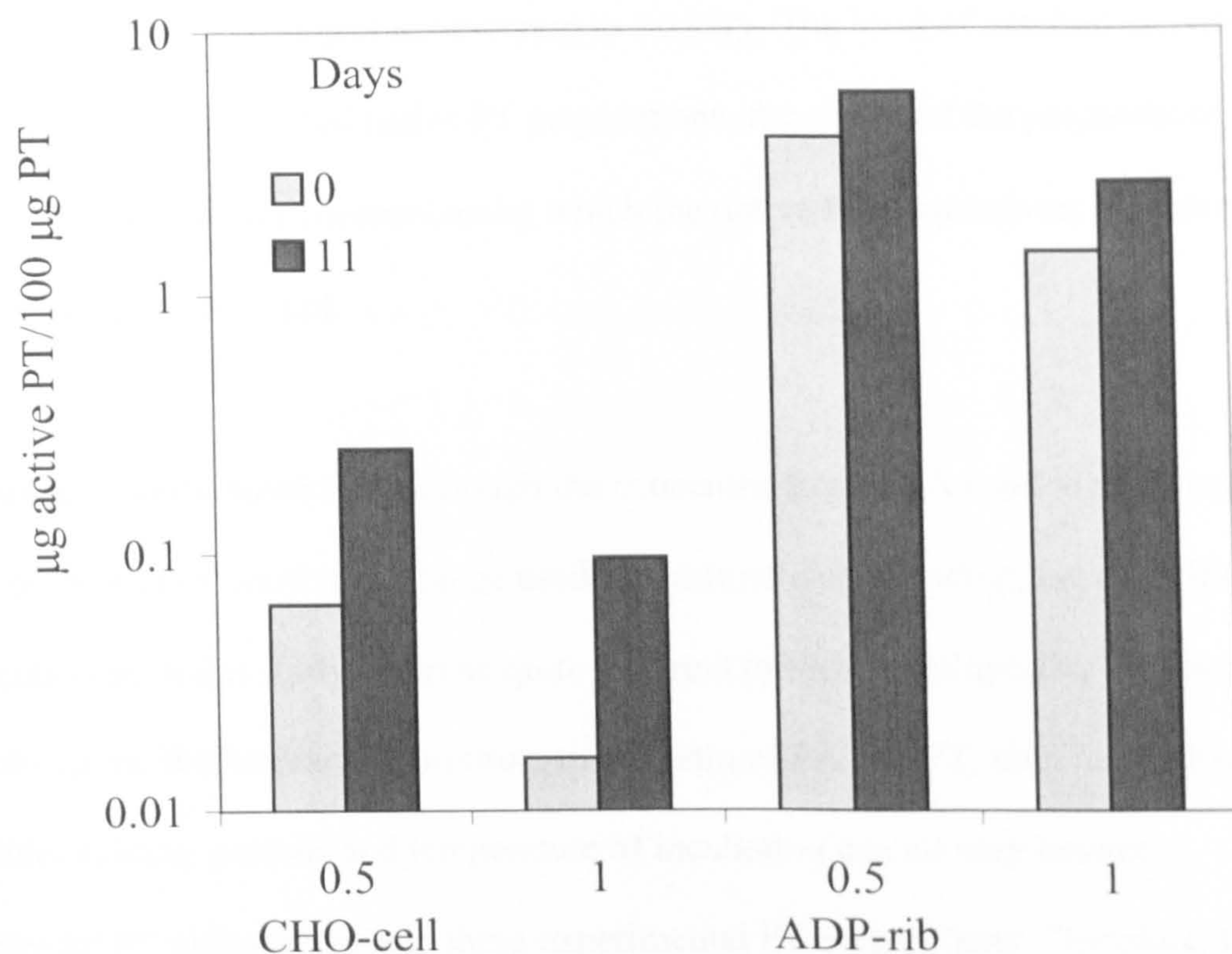


Figure 7.7 Reversion to toxicity of FA-treated native PT

The quantity of PT with ADP-ribosyl transferase activity and CHO-cell toxicity, in native pertussis toxin (PT) preparations treated with 0.5 and 1.00 % (w/v) FA, was determined before and after incubation for 11 days at 37 °C. The ADP-ribosylation assay measured the ribosylation of a fluorescent- tagged peptide substrate using an enzymatic-HPLC coupled assay (Cyr et al., 2001). The CHO-cell toxicity assay measured the ability of the preparations to cause CHO-cell clumping, performed according to Gillenius et al., 1985. The amount of active PT (µg) in each preparation was determined relative to reference PT (code 90/518, NIBSC reagent) and presented as a proportion of 100 µg total protein in the sample.

that FA-treated native PT, under the conditions of preparation in this study, contains residual activity and is subject to reversion to toxicity. The level of residual activity harboured by the FA-treated native PT preparations, the ability of the preparations to revert to toxicity and the mechanism by which the native PT preparations are detoxified are now discussed in detail.

It should be clearly noted that, although the concentrations of FA used to treat native PT in the present study were in the range used for commercial toxoiding, the detoxification protocols used in this study could be quite different to those employed by the vaccine manufacturers. Buffer reaction mixture, molar ratio of FA and PT, intervals of FA additions, mixing, periods and temperature of incubation can all vary between commercial PT preparations and these experimental PT preparations. Therefore, toxicity measurements obtained in this study are unlikely to be indicative of the toxicity of commercial PT antigens prepared with the same final concentration of FA.

Residual toxicity

The present study revealed that all of the FA-treated native PT preparations harboured measurable residual activity as detected by both the *in vivo* and *in vitro* tests employed. The histamine sensitisation and leukocyte proliferation tests, both suggested that there was over 2 µg of active PT per 100 µg of protein in even the highest FA-treated (1.0 %) PT preparation. The lowest FA-treated (0.25 % FA) preparation retained 1 µg of CHO-cell toxic PT and 5.72 µg of ADP-ribosylating PT, and the highest FA treated PT (1.0 % FA) still harboured 0.053 µg CHO-cell toxic PT and 1.48 µg ADP-ribosylating PT per

100 µg protein. In addition, the higher toxicity measured with *in vivo* compared with *in vitro* assays may also suggest that there was reversion to toxicity *in vivo*.

Toxicity reversion

Reversion to toxicity of the FA-treated native PT samples was indicated by both *in vivo* and *in vitro* studies. The first clue of reversion was from observations of mice fatalities in the low FA-treated native PT preparations, ten days after immunisation. Subsequently it was found that leukocyte proliferation and histamine sensitisation activities were much higher than would be expected from comparison with the *in vitro* assays, which had used the same reference PT. After incubation at body temperature for 11 days, the *in vitro* assays detected a much higher toxicity than before incubation, providing direct evidence for toxicity reversion.

The reversion data supports previous results from a model for reversion to toxicity (Quentin-Millet *et al.*, 1988). The progression of leukocytosis was measured in nude mice, which are incapable of producing neutralising antibodies that may delay or mask the effects of reversion. It was found that the maximum leukocyte count induced by a FA-treated native PT preparation was not detected at 3 days as for untreated native PT (Munoz and Bergman, 1977) but at six days post-immunisation, and that a considerably elevated count was still observed for 16 days after the maximum leukocyte count. This study concluded that there was reversion to toxicity of the PT preparation and that there may have been slow release of the native toxin, which prolonged the leukocytosis.

In overall analysis, it is conceivable that *in vitro* assays may not reveal the full *in vivo* toxicity of some PT toxoids, highlighting a potential problem in the sole use of *in vitro* tests for the realistic assessment of the toxicity of pertussis vaccine toxoids in humans.

Mechanism of detoxification

The present study further uncovered that the FA-induced decrease in the *in vitro* and *in vivo* toxic properties of native PT was due to modifications in both the S1 catalytic site and the B-oligomer cell-binding domain. The observed reduction in the ADP-ribosyl transferase activity (by cell-free assay) established that there was specific FA-induced modification of the S1 catalytic site. The greater decrease in CHO-cell toxicity (95 %) compared with ADP-ribosyl transferase activity (74 %), with 1.00 % FA-treatment, illustrated that the B-oligomer binding domain was also FA-modified.

In accord with these results, evidence for FA-induced modification of the S1 subunit has previously been shown in the decrease of specific mAb (1B7) affinity constant from 5×10^7 L/mole, for untreated mutant PT, to an undetectable level with 0.052 % (w/v) FA treated mutant PT (Nencioni *et al.*, 1991). In addition, specific modification of the B-oligomer has also been demonstrated by a 2-fold reduction in the haemagglutination activity of mutant PT after treatment with an unspecified concentration of FA (Nencioni *et al.*, 1990; Nencioni *et al.*, 1991)

Moreover, the present study demonstrated that the mechanism of reversion to toxicity is by reversal of these FA-induced modifications in both the S1 catalytic site and the B-

oligomer cell-binding domain. After incubation for 11 days at 37 °C the CHO-cell toxicity of the 1.00 % FA-treated native PT preparation increased by almost twice as much as the ADP-ribosyl transferase activity. This greater increase in CHO-cell toxicity compared with ADP-ribosylation activity demonstrated that FA-induced modifications were reversed in a proportion of B-oligomers as well as S1 subunits.

The overall aim of this study was to investigate the relationship between the molecular structure and immunogenicity of PT. A molecular model of PT was developed and corroborated using solution techniques. For the majority of the study, the size, conformation and epitope integrity of five commercial toxoids and a series of FA-treated native and mutant PT preparations (0 – 1.0 %, w/v, FA) were evaluated. In addition, the toxicity and immunogenicity of the PT preparations was assessed by *in vivo* and *in vitro* methods.

8.1 Molecular modelling and physico-chemical analysis of PT highlight novel structural features

Computer modelling and analysis of PT in solution revealed some intriguing new insights into the structure of PT. Results from analytical ultracentrifugation demonstrated that PT may exist in equilibrium between holotoxin and separated A and B-oligomer.

In addition, a combination of fluorescence, circular dichroism and computer modelling uncovered that native PT and the catalytically inactive genetic mutant PT, PT-9K/129G, are structurally comparable. Interestingly, both forms of PT can bind NAD^+ , as shown by fluorescence studies.

Computer modelling and fluorescence spectroscopy demonstrated that structural adjustment following ‘catalytic activation’, by cleavage of the disulphide bond Cys-41 – Cys-210, is required for binding of NAD^+ in the active site of the S1 subunit. An ‘activated’ NAD^+ -bound model of PT was produced, and demonstrated that further structural changes occur on NAD^+ binding to reveal a G-protein binding site. This novel molecular model could provide the basis for further studies on the structure and function of PT.

8.2 Different chemical treatments of PT alter the molecular structure and immune response

From studies of five commercial PT toxoids some interesting comparisons can be made between the molecular structure and immunogenicity of PT. The five PT toxoids were detoxified with different chemicals, and included the mutant PT, which is stabilised with 0.035 % (w/v) FA (Table 8.1). A degree of cross-linking and aggregation was not detrimental to the anti-PT immune response in mice. This was demonstrated by the slightly aggregated mutant PT treated with 0.035 % (w/v) FA, and highly cross-linked and aggregated glutaraldehyde plus FA-treated native PT. Both of these toxoids induced good protection against aerosol infection and higher total IgG antibody responses compared with untreated PT, which was not cross-linked or aggregated (Table 8.1). Results of glutaraldehyde-treated PT and tetranitromethane-treated PT showed that much higher aggregation correlated with a decrease in antibody response and protection.

Table 8.1 Summary of thesis results for untreated PT, 0.25 and 1.00 % formaldehyde (FA)-treated mutant and native PT preparations, and five commercial PT toxoids

The majority of the results described throughout the thesis are summarised, by rationalising the raw data as + and – symbols (detailed in the legend). For the 0.25 and 1.00 % FA-treated PT preparations, the cells are split where different results were obtained for mutant and native PT.

Property	PT Toxoid							
	Untreated	0.25 % FA	1.00 % FA	Commercial PT toxoids				
		Native Mutant	Native Mutant	^h Mutant PT, 0.035 % formaldehyde	Glutar- aldehyde plus Form- aldehyde	Glutar- aldehyde	Tetranitro- methane	Hydrogen peroxide
Unfolding ^a	-	++	+++	+	++	*	*	*
Cross-linking ^b	-	+++	+++	nd	+++	+++	nd	-
Aggregation ^c	-	++	+++	+	++	+++	+++	-
Anti-S1 binding ^d	+++	+	nd	nd	++	++	nd	+
Anti- B-oligomer binding ^e	+++	+	nd	nd	+	+	nd	-
Total IgG response ^f	+	+/++	+/+++	+++	+++	++	++	+
Neutralising antibodies ^g	nd	+/+++	+/+++	nd	nd	nd	nd	nd
IgG1/IgG2a ^h	+	+/+	+/+	+++	+	++	+++	++
Protective efficacy ⁱ	nd	nd	nd	+++	++	+	-	-

Table 8.1 (continued)

nd: experiment not done

^a**Extent of unfolding**; by Fmax of fluorescence spectroscopy: 337 nm = - ; 338 – 340 nm = +; 340 – 342 nm = ++; above 342 nm = +++; * = fluorescence spectrum shape is atypical

^b**Extent of cross-linking**; based on highest Mr detected by mAb in western blots: < 60 kDa = - ; > 60 kDa = +; > 100 kDa = ++; 250 kDa, gel limit, = +++. 0.25 and 1.00 % FA-treated PT were not analysed so results based on 0.1 and 0.5 % FA-treated PT, which included species of at least 250 kDa

^c**Extent of aggregation**; based on SEC profile showing ½ or more of protein: eluting before 13 mL = +++; eluting before 16 mL = ++; eluting before 17 mL = +; eluting at ~ 18 mL = -

^{d,e}**Extent of antibody binding**; based on comparative densities of mAb binding in western blots (for commercial toxoids), or extent of mAb-binding in ELISA's (for experimental formaldehyde-treated preparations): no data available for the 0.25 or 1.00 % FA-treated preparations, therefore results presented for 0.25 % formaldehyde-treated sample was obtained for the 0.1 % FA-treated preparation.

^f**Total IgG response**; Relative geometric mean total IgG potency: < 1 = +; 1 – 3 = ++; > 3 = +++

^g**Neutralising antibodies**; relative geometric mean neutralising titre in CHO-cells: < 200 = +; > 200, < 300 = ++; > 300 = +++

^h**IgG1/IgG2a ratio**; ratio of relative geometric mean potencies: < 1 = +; > 1, < 10 = ++; > 10 = +++

ⁱ**Protective efficacy**; Geometric mean CFU per lung: > 500 000 = -; > 200 000, < 500 000 = +; > 20 000, < 200 000 = ++; < 20 000 = +++

^j**Mutant PT toxoid**; Mutant PT (PT-9K/129G) is treated with 0.035 % formaldehyde, in order to stabilise the protein (Nencioni, L. *et al.* 1991)

However, these toxoids still induced higher antibody responses than the untreated PT (Table 8.1). In contrast to the other toxoiding chemicals, hydrogen peroxide treatment did not cause any cross-linking or aggregation but induced the poorest antibody response and protection (Table 8.1).

Although some molecular aggregation was beneficial, the loss of local structure was unfavourable in maintaining a good immune response. This was demonstrated by toxoids with an atypical fluorescence spectrum shape, namely glutaraldehyde-, tetranitromethane- and hydrogen peroxide-treated PT, which induced relatively low antibody responses and protection against aerosol infection. Higher responses were mounted in response to the 0.035 % FA-treated mutant PT and glutaraldehyde plus FA-treated PT toxoids, which both maintained a fluorescence spectrum shape characteristic of untreated PT. Atypical fluorescence spectra may indicate gross amino acid modification and thus widespread loss of immune epitopes. Atypical fluorescence was indeed found to correlate with a loss in the protective S1 epitope (Table 8.1).

The type of immune response induced by the commercial toxoids, as determined by the IgG1 : IgG2a ratio, did not directly correlate with the extent of aggregation, cross-linking or unfolding (Table 8.1). Other physico-chemical factors, such as specific amino acid alteration or biological properties, such as toxic activity, may direct changes in the type immune of response. It is intriguing that two highly immunogenic toxoids, 0.035 % FA-treated mutant PT and glutaraldehyde plus FA-treated PT toxoids appeared to induce

opposite immune response types, Th2 and Th1 respectively. Cytokine studies of these immune responses should be carried out to confirm these results.

In this study the total IgG antibody response correlated with protection against aerosol infection in mice. These results are an indication that circulating antibodies may protect soon after immunisation, in this case within a five-week immunisation period. However, there is ongoing debate about whether antibody response to PT is a valid indication of protection. Studies have shown that patients with circulating antibody are more likely to be protected against whooping cough (Zackrisson, Taranger, and Trollfors, 1990; Isacson *et al.*, 1994) but that the titres do not always necessarily correlate with protection (Ad Hoc Group for the Study of Pertussis Vaccines, 1988; Tran Minh *et al.*, 1999). There is evidence that cellular responses are important for protection (Mills *et al.*, 1993; Mills and Redhead, 1993; Mills *et al.*, 1998; Ryan *et al.*, 1997a), but these were not directly assessed in this investigation. However, the very different IgG1 : IgG2a ratios of the protective toxoids, 0.035 % FA-treated mutant PT and glutaraldehyde plus FA-treated PT, supports the current notion that both cellular and humoral responses may be important for protection.

The results from this comparative study of toxoids suggest that aldehydes, tetranitromethane and hydrogen peroxide induce a variety of structural modifications. A low level of aggregation and cross-linking is beneficial to the immunogenicity of PT toxoids and even very high levels of aggregation appear to be much better than no aggregation at all. However, large changes in the local protein structure were detrimental

to the immunogenicity of PT. In addition, these different chemical-induced modifications appear to alter the type of immune response. Biological characteristics such as residual toxicity may also have played a part in shaping the immunological properties of these toxoids.

8.3 FA-induced cross-linking, aggregation and amino acid modification of PT modulates the immune response

Studies designed to probe the effect of a controlled increase of FA-treatment on both mutant and native PT afforded a more detailed description of FA toxoiding. In a concentration-dependent fashion, FA induced PT aggregation, inter-subunit cross-linking and unfolding. The principal chemical modifications are likely to be methylene bridges between FA-reactive amino acids within the PT molecule and amino acid modification by lysine cross-linking from the reaction mixture (Tome and Naulet, 1981; Tome, Kozlowski, and Mabon, 1985). These cross-links have been found to be either stable, acid labile or reversible (Bizzini and Raynaud, 1974; Tome *et al.*, 1979). FA-modified amino acids may also change the charge or hydrophobicity of molecular surfaces, altering their association. A low concentration of FA (0.035 %, w/v) appeared to stabilise the holotoxin by inter-subunit cross-linking between neighbouring subunits in the native structure, preventing their dissociation. Additionally, at this low FA-concentration a small quantity of high molecular weight species was produced, associated by reversible interactions. High concentrations of FA (≥ 0.50 %, w/v) caused less uniform cross-linking, gross

chemical modification of the amino acid residues and the formation of heterogeneously sized molecular aggregates.

Structural correlates of immunogenicity were assessed for the mutant PT preparations. Immunological results from native PT, however, could not be interpreted in the same way, due to the significant immunogenic effects of residual quantities of active PT.

As found for the commercial toxoids, some aggregation was beneficial to the immunogenicity of PT. This was demonstrated by the higher IgG responses induced by 0.25 and 1.00 % FA-treated mutant PT preparations compared with untreated PT (Table 8.1). These antibody responses were higher despite the destruction of A-subunit and B-oligomer epitopes (Table 8.1). These results suggest that some FA-induced modifications are beneficial for the induction of a high IgG response. This may be due to increased *in vivo* stability of the toxin, and indeed FA-induced cross-linking was found to prevent holotoxin dissociation *in vitro*. It is also possible that intact epitopes are efficiently presented to the immune system on high molecular weight PT molecules.

Although the quantity of total IgG antibodies induced was not reduced by FA treatment of mutant PT, the quality of response clearly was. This was demonstrated by the observation that the number of antibodies of neutralising quality declined as a function of FA (Table 8.1). This may have been due to the alteration of important protective structural epitopes on the surface of the molecule. The decline observed in A-subunit and B-oligomer epitopes by mAb-binding *in vitro* indicates this. In addition the IgG1: IgG2a

ratio increased with FA treatment (Table 8.1), suggesting the immune response had shifted towards a more Th2-type response. It is likely that the size of the molecules and their chemically modified surfaces affects their interaction with antigen presenting cells. In addition, the cross-linking of the FA-treated species is likely to effect the processing of antigen, by antigen presenting cells. Therefore, the FA-induced modifications could lead to a change in the cytokine environment. It has been demonstrated that antigen size can effect the differential induction of Th1 and Th2 responses by altering the pattern of cytokine secretion by the antigen presenting cells (Brewer *et al.*, 1998). In addition, a different study demonstrated that FA-treatment of a variety of *B. pertussis* antigens altered the proliferation of antigen-specific T-cell clones. The study concluded that this was likely to be a result of altered proteolytic processing by the antigen presenting cells influencing antigen presentation to T-cells (Di Tommaso *et al.*, 1994).

8.4 Detoxification and toxicity reversion of FA-treated PT is by modification to both the A- and B- functional domains

The effect on the toxic biological activities of native PT with increasing FA-treatment was investigated by two *in vitro* tests; a CHO-cell toxicity assay and a cell-free ADP-ribosylation assay. For CHO-cell toxicity PT molecules require both a functional A- and B-domain. The B-domain facilitates entry into the CHO-cell, while the A-domain exerts the toxic effects. For ADP-ribosyl transferase activity in the cell-free system the PT molecule only required a functional A-domain. The greater decrease in the CHO-cell

toxicity compared with the ADP-ribosyl transferase activity, with increasing FA, demonstrated that both the A and the B-functional domains of PT were modified by FA-treatment.

These assays were also used to detect reversion to toxicity of the FA-treated native PT preparations. After incubation at body temperature for 11 days, a greater increase in CHO-cell toxicity compared with ADP-ribosyl transferase activity indicated that FA-induced modifications to both A and B functional domains were reversed.

It is likely that the detoxifying modifications to the A and B domains are a consequence of the FA-induced cross-linking and heterogeneous aggregate formation, as characterised in detail for FA-treated mutant and native PT. The detoxifying modifications could be due, at least in part, to specific FA-induced cross-linking between amino acids composing the functional domains or by the cross-linking of lysine from the reaction mixture.

Another mechanism for detoxification may include burying of the functional domains in aggregates.

It is interesting to speculate that reversion to toxicity could occur by the reversal of these mechanisms. Some FA-induced cross-links have been found to be acid labile and reversible (Bizzini and Raynaud, 1974; Tome *et al.*, 1979). In addition, non-covalent association such as hydrophobic and charge interactions may be readily disrupted.

Disruption of unstable cross-links within functional domains could render them active again. Large complexes harbouring unmodified functional domains may undergo

unfolding and dissociation, also releasing active molecules. Indeed some aggregates detected by FPLC and SEC-MALLS could not be detected by the denaturing technique of SDS-PAGE, suggesting a degree of dissociation.

8.5 Active PT can both suppress and augment the immune response

Assessment of the toxic biological activities of FA-treated native PT revealed evidence of residual toxicity and identified reversion to toxicity of the preparations. It follows that this difference in toxicity between the native and mutant PT preparations may have contributed to the large differences observed in the extent and type of immunogenicity they induced. The results demonstrated that, relative to the corresponding mutant PT preparations, the most toxic native PT preparation inhibited the immune response, while much less toxic preparations augmented the response.

The lowest FA-treated (0.25 %, w/v, FA) native PT preparation, in this experiment, harboured the highest levels of residual toxicity (1 µg active PT / 100 µg protein, by CHO-cell assay before incubation), and indeed induced a significantly lower total IgG antibody response compared to the corresponding mutant PT preparation. It is very likely that the high residual toxicity caused the suppression of antibody production. There is some previous evidence that pertussis toxin can cause immune-suppression to different co-administered antigens (Asakawa, 1969). In this study a marked decrease in mouse

antibody response to sheep red blood cells was discovered when the mice were pre-injected with active pertussis toxin. The antibody response was decreased significantly with a dose of 0.6 µg of active PT. This is in direct correlation with the concentration range of active PT found to inhibit anti-PT total IgG in this investigation. That is 1 µg of active PT was inhibitive and 0.1 µg of active PT was stimulatory.

In contrast, the 1.00 % FA-treated native PT had much less residual toxicity (0.16 µg active PT / 100 µg protein, by CHO-cell assay before incubation), and the total IgG response was three-fold higher than for the corresponding mutant PT preparations. The proposal that these augmented responses were induced by residual amounts of active PT, concur with the known immune-enhancing properties of PT. Early work in this area showed that PT had the ability to enhance the IgE antibody responses to coadministered antigen (Munoz and Bergman, 1977). In addition PT, at low concentrations of 0.2 – 0.5 µg/dose, induces enhanced protection against intra-cerebral *B. pertussis* infection when co-administered with other *B. pertussis* antigens such as FHA and fimbriae. It has also been demonstrated that trace amounts of PT (0.2 µg/dose) stimulate proliferation of murine interleukins, in response to lysozyme (Shive *et al.*, 2000).

Additionally in this study, an increase in FA-treatment, and thus a decrease in the residual toxicity, of native PT was found to correspond with a decrease in the IgG1 : IgG2a ratio. The response induced by 1.00 % FA-treated native PT yielded a markedly lower IgG1 : IgG2a ratio compared with the corresponding mutant PT, implying that the response was more Th1 polarised. In correlation with this, trace amounts of PT (0.2 µg/dose) have been

shown to preferentially induce IgG2a subclass antibodies and other Th1 markers such as interferon- γ cytokines (Shive *et al.*, 2000). It has been demonstrated that small concentrations of active PT (0.12 μ g/dose) administered with heat-treated whole cell *B. pertussis* vaccine induced a huge increase in nitric oxide production by murine macrophages, which was associated with better protection against intracerebral and aerosol challenge with *B. pertussis* (Xing, Canthaboo, and Corbel, 2000).

Since the immune suppression and enhancement by FA-treated native PT preparations is relative to that by the catalytically inert (mutant) PT preparations, this study suggests that both suppression and enhancement of the murine immune response is ADP-ribosyl transferase-dependent.

ADP-ribosylation-dependent immune enhancement by PT is supported by studies which show that in lymphocyte cultures, native PT but not PT-9K/129G can promote T-cell dependent production of IgE by B-cells (Pouw-Kraan *et al.*, 1995). In addition, enhancement of the murine interleukin response to lysozyme by native PT could not be reproduced with the same concentration of B-oligomer alone (Shive *et al.*, 2000). It has been speculated that the active PT stimulates antigen-presenting cells via ADP-ribosylation of their G-proteins leading to enhanced cytokine secretion (Shive *et al.*, 2000).

The ADP-ribosylation of host G-proteins leads to the constitutive action of adenylate cyclase and thus the continued production of cyclic adenosine monophosphate (cAMP). It

is conceivable that native PT both inhibits and enhances the immune system via the immunoregulatory effects of cAMP. This messenger molecule is produced in immune cells and is an essential factor in modulating immune responses (Koh, Yang, and Kaminski, 1995; Bell and Brunton, 1986; Bihoreau *et al.*, 1991; Kvanta *et al.*, 1990). The concentration of cAMP induced by residual PT activity was not investigated in this study. However, the literature suggests that immunomodulation by cAMP is biphasic (inhibitive and stimulatory), which may explain the apparent inhibition and stimulation of antibody response caused by active pertussis toxin.

At a high concentration ($> 250 \mu\text{M}$) cAMP is known to inhibit T-cell responses, as determined by inhibition of Ca^{2+} mobilization, PIP2 hydrolysis, interleukin (IL)-2 and IL-2 receptor expression (Lerner, Jacobson, and Miller, 1988; Anastassiou *et al.*, 1992; Imboden *et al.*, 1986; Novak and Rothenberg, 1990; Kammer, 1988; Krause and Deutsch, 1991) and IL-2 dependent T-cell proliferation (Johnson, Davis, and Smith, 1988). These results correspond with those of the most toxic PT preparation (0.25 % FA) which also induced a suppressed immune response that appeared to be Th2-polarised (Table 8.1).

In contrast, at lower concentrations (10 – 100 μM), cAMP was found to be stimulatory to humoral immune responses and lymphocyte proliferation (Koh, Yang, and Kaminski, 1995). It has also been shown to enhance IL-2 production (Chakkalath and Jung, 1992) and IL-2 receptor expression (Scholz and Altman, 1989). These findings correlate with the Th1-type immune enhancement observed with low levels of active PT in the 1.00 % FA-treated native PT preparation.

These studies cannot rule out the possibility of intrinsic immune-enhancing properties of the B-oligomer, as has been indicated by studies of the structurally related cholera toxin and heat labile enterotoxin (Williams, Hirst, and Nashar, 1999), but they do suggest a specific immune enhancing role for the catalytic S1 subunit.

Cross-linked molecular aggregates of native PT appear to be advantageous for immunity. They form a vehicle for the slow release of native structure *in vitro*, which may liberate protective epitopes, but on the other hand leave the toxicity of the preparation unpredictable. Progress continues to be made in the vaccine field, not least by the increasingly detailed descriptions of purified antigens, but the balancing act continues. Further studies on the physico-chemical nature of immunogenic molecules and immunogenic markers of protection are required for further progress in this area. These studies could lead to the selection of molecules that can direct the immune response, and could also extend to the development of *in vitro* studies as alternatives to the use of animals.

Appendix 1

Information for the secondary structure of PT (PDB code 1PRT, (Stein *et al.*, 1994a)) was obtained from the Research Collaboratory for Structural Bioinformatics

<http://www.rcsb.org/pdb/cgi/explore.cgi?job=chains&pdbId=1PRT&page=&pid=26982997264039>, and is predicted using an implementation of the Kabsch and Sander method (Kabsch and Sander, 1983).

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